PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF *Staphylococcus aureus* ISOLATED FROM CLINICAL SPECIMENS AND FOOD ARTICLES OF ANIMAL ORIGIN

THESIS

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ABSTRACT

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Staphylococcus aureus is a well known pathogen of man and animals. Despite the development of antimicrobial agents the staphylococcal infection still remains an important cause of morbidity and mortality. The spectrum of disease produced by this organism varies from pyogenic infections to toxin-mediated phenomenon. It also causes serious infections in man including deep-seated abscesses, bacteraemia, endocarditis, osteomyelitis, food poisoning etc. The main reservoirs of infection are infected cases and carriers who spread infection in a hospital or in a community through droplets in air or through fomites. Carriage in nose plays an important role in its epidemiology and pathogenesis.

In animals too it causes a wide spectrum of diseases like intramammary infections in lactating animals, sporadic septicaemia in pigs, abscesses, synovitis, arthritis, osteomyelitis, dermatitis in poultry and ducks. Bovine mastitis is one of the most important bacterial diseases in dairy cattle throughout the world, and it is responsible for great economical losses to milk producers as well as to the milk processing industries. *S. aureus* can also multiply in many food items.

Various global studies are available to characterize the *Staphylococcus aureus* at molecular level, however, the studies are fragmentary from India on the current aspect and especially in *Staphylococcus aureus* isolates obtained from samples of animal origin. Therefore, the present study was undertaken with the aims to evaluate the phenotypic and genotypic characters of *Staphylococcus aureus*, whicl might provide an understanding of the distribution of the clones and might aid in the development of the steps to control *S. aureus* infections in humans as well as in animals.

A total of 102 *Staphylococcus aureus* from human clinical specimens and 100 from animal-originated (both animal-clinical and animal-originated food) samples were randomly selected for the study. All were tested phenotypically according to the standard methodology and confirmed as *S. aureus*.

Among 102 **human clinical isolates,** maximum number of isolates were resistant to penicillin (98.03%) followed by cotrimoxazole (69.61%), tetracycline (68.63%), amoxycillin (64.7%), ciprofloxacin (60.79%), erythromycin (54.9%), amikacin (35.3%), **oxacillin (32.35%),** cefaclor (32.35%), ceftriaxone (32.35%), ceftazidime (32.35%), cefepime (32.35%), chloramphenicol (23.53%) and gentamicin (27.45%). While none of the isolates were found resistant to vancomycin and teicoplanin. Out of these 102 **clinical isolates,** 80 (78.4%) were β-lactamase producers as identified by iodometric method.

The isolates from **animal-origin samples** showed resistance to penicillin (93%), erythromycin (51%), tetracycline (49%), ciprofloxacin (39%), cotrimoxazole (54%), chloramphenicol (34%), amikacin (27%), **oxacillin (27%),** cefaclor (27%), ceftriaxone (27%), ceftazidime (27%), cefepime (27%), gentamicin (20%), and amoxycillin (19%). Only one isolate was found susceptible to all 16 antibiotics and none of the isolates was found resistant to vancomycin and teicoplanin. Out of 100 **animal-origin isolates**

69 (69%) were found β-lactamase producers by iodometric method. Interestingly, both human and animal isolates showed multidrug resistance patterns.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major nosocomial pathogen worldwide and is often difficult to detect due to the heterogeneous nature of expression of oxacillin resistance. All the human and animal isolates were tested for oxacillin resistance by disc diffusion method, screen agar plate method, and for detection of *mec* A gene by PCR. Out of 102 **human clinical isolates,** 33 were oxacillin resistant by disc diffusion method, 32 by screen agar plate method and 34 isolates were found positive for *mec* A gene by PCR whereas 29% isolates from **animal-origin samples** were identified as MRSA by PCR, 27% by disc diffusion method and 26% by oxacillin agar plate method. The results of our study on PCR of MRSA seem to be promising for early and reliable identification of MRSA.

Bacteriophage typing was performed in 102 **human clinical isolates**. Out of these isolates tested, only 55 (53.9%) isolates could be typed by the conventional set of phages at RTD. 47 non-typeable isolates were further tested at 100 RTD, they showed 16.67% more typeability. The total typeability at 1 RTD and 100 RTD was observed in 70.58% of clinical isolates including 10 methicillin-producing strains.

MRSA phage groups were used to type methicillin-resistant *Staphylococcus aureus* isolates of clinical specimens. A total of 14 (41.18%), out of 34 MRSA, were found typeable at RTD and 100 RTD using MRSA phages. The non typeable isolates at RTD could be typed at 100x RTD and on addition 16.6% isolates were typed. Out of 100 **animal-origin isolates** none of the isolates were typeable at routine test dilution and at 100 RTD. Only two isolates (one from raw milk and the other from buffalo meat) could be typed by using MRSA phage groups at 100 RTD which belonged to phage group II.

All 202 *Staphylococcus aureus* isolates were found positive for the presence of thermostable nuclease (*nuc)* gene by polymerase chain reaction.

Coa gene was detected by PCR and twelve different electrophoretic patterns were observed in human clinical isolates, whereas 7 electrophoretic patterns were observed in animal-originated isolates.

The **molecular typing** of the **human-clinical isolates** and **animal-origin isolates** was carried out by **Coa-RFLP**, after the digestion with *Alu 1* enzyme. Twelve types of ***coa* -RFLP** patterns were observed in **human-clinical isolates**. Among these 12 patterns, 7 group patterns, namely, group I, group II, group IV, group V, group VI, group VIII and group XI were noticed in MRSA isolates. Rest of the group patterns were in MSSA isolates. In 100 **animal-origin isolates,** 7 types of ***coa* –RFLP** patterns were observed. Four group patterns, namely, group II, group III, group I, and group VII, were noticed in 29 MRSA isolates from animal-origin samples. Rest of the group patterns were in MSSA isolates. In the present study, *S. aureus* isolates from animal-origin samples and human clinical specimens were found to differ in their gene patterns whereas animal clinical specimens and animal-origin food samples were showed mixed Coa-RFLP gene patterns. Antibiotic resistance patterns in relation to *Coa* – RFLP pattern could not be inferred in isolates obtained from food samples due to variability of resistance patterns; 40 different types of patterns.

We feel that *Coa*-RFLP technique could be incorporated as diagnostic tool to confirm the MRSA. Since the *Coa*-RFLP patterns of the MRSA strains were unique and distinct from the MSSA strains from both human and animal isolates of *Staphylococcus aureus*. *Coa*-RFLP is performed with primers, homologous to a conserved region with in the *coa* gene, in order to amplify the sequence encoding the C-terminals region of this molecule. Since the number of repetitive sequence varies within the *coa* gene, the resulting PCR products of individual strains can be of different lengths. Therefore we found different length and different patterns of *S. aureus* strains in *coa* RFLP. It is noted that there is extensive polymorphism with *coa* gene circulating in human and animal strains. We believe that the heterogeneity observed for the *coa* gene has a potential discriminatory power for further epidemiological studies of medical and veterinary importance.

For the detection of enterotoxin genes. namely enterotoxin A, enterotoxin B and enterotoxin C, PCR assays were used. A total of 202 isolates (102 human clinical isolates and 100 animal-origin isolates) were tested for the production of enterotoxin A, B and C. Of the 102 **human-clinical isolates,** 3 were found enterotoxigenic, out of which one stool sample showed presence of enterotoxin A, one vomitus sample showed enterotoxin B while one urine sample showed enterotoxin C.

Out of 100 **animal-origin isolates,** a total of 13 were found enterotoxigenic. Out of which 2 were positive for enterotoxin A (1 from animal clinical raw milk sample and 1 from buffalo meat); 3 were positive for enterotoxin B (1 from animal clinical raw milk sample, 1 from a sweet, and 1 from cottage cheese) and 8 were found positive for enterotoxin C (1 from animal clinical raw milk, 1 from paneer, 3 from goat meat, and 3 from buffalo meat).

Conclusions:

On the basis of this study, following **conclusions** were drawn:

- Multidrug resistance was noticed in both human clinical and animal-origin isolates.
- None of the isolate was found resistant to vancomycin and teicoplanin in both clinical and food isolates.
- Phage typing of the **clinical isolates** showed that only 70.58% clinical isolates were typeable using conventional phages at 1 RTD and at 100 RTD including 10 methicillin-producers. Amongst these, maximum number (35.3%) of isolates belonged to phage group III.
- Majority of the MRSA isolates were found non-typeable by conventional sets of phages. By using a set of MRSA phages they showed a typeability of 41.8% (14/34).
- In **animal-origin**, only 2% isolates were found typeable using MRSA phages at 1 RTD and 100 RTD both of them belonged to group II.
- MRSA were detected in **human-clinical isolates** by disc diffusion method in 32.35%, by screen plate agar method in 31.41% and by PCR in 33.33%.
- 29% isolates from **animal-originated samples** were identified as MRSA by PCR, 27% by disc diffusion method and 26% by oxacillin agar plate method.

- All the coagulase positive *Staphylococcus aureus* isolated from clinical and animal-origin samples were found Tnase and Nuc gene producers.
- 12 Coa-RFLP patterns were observed in **human clinical isolates**. Whereas 7 types of *coa* –RFLP patterns were observed in **animal-origin samples**. Knowledge about the genetic variability within different populations may help in the identification of the most likely source of an isolate.
- Coa-RFLP patterns were different for **clinical and animal-originated food isolates** and suggest a divergence between *Staphylococcus aureus* isolates of human and bovine origin.
- Results of coagulase gene typing demonstrated that the MRSA and MSSA strains from **clinical specimens** could be grouped into 7 and 5 *Coa*-RFLP patterns, respectively. However, in **food isolates** 4 from MRSA and 3 from MSSA *Coa*-RFLP patterns were observed.
- The MRSA and MSSA strains did not share similar PCR-RFLP patterns and this could be initialized as a diagnostic tool to differentiate MRSA from MSSA. It offers an attractive option to be considered for rapid epidemiological analysis of *S. aureus* strains.
- 102 **human clinical isolates** were tested for commonly encountered enterotoxins; three of them were found enterotoxin producers, out of which 1 was positive for enterotoxin A, 1 for enterotoxin B and 1 for enterotoxin C.
- Out of 100 **animal-origin isolates**, a total of 13 were found enterotoxogenic. Of which 2% were positive for enterotoxin A; 3% were positive for enterotoxin B and 8% were found positive for enterotoxin C.

In nutshell, diversity between clinical and food isolates of *Staphylococcus aureus* was noticed and that the incidence of methicillin resistance was quite high in this collection of isolates. Concomitant high resistance to other classes of antibiotics was also noted. Phage typing was found to be of low discriminatory value whereas Coa-RFLP could discriminates a fairly large numbers of bacterial isolates and suggest that Coa-RFLP could be used as an epidemiological typing method for *Staphylococcus aureus.* Enterotoxin A and B were detected in our collection of clinical isolates, whereas all three enterotoxins were prevalent in food isolates with enterotoxin C being the predominant type.

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Certificate

Certified that the thesis entitled **"Phenotypic and Molecular Characterization of *Staphylococcus aureus* isolated from clinical specimens and food articles of animal origin"** submitted to the Aligarh Muslim University, Aligarh, in fulfilment of the requirement for the award of the degree of **Doctor of Philosophy**, embodies the original research work carried out by **Anju Tyagi,** under our guidance and supervision. The work has not been submitted elsewhere in part or full for the award of any other degree of this or any other university.

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DEDICATED TO

My
Papa ji
&
Mummy

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"To begin with the name of maa Durga, the most beneficent and merciful"

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Anju Tyagi

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Abbreviations

BHI	-	Brain Heart Infusion
BORSA	-	Borderline resistant *Staphylococcus aureus* strains
Cham cham	-	A type of sweet prepared from milk
Coa	-	Coagulase gene
coa	-	Coagulase gene
Conj. Swab	-	Conjunctival swab
CRF	-	Coagulase reaction factor
CSF	-	Cerebrospinal fluid
Cx. swab	-	Cervical swab
°C	-	Degree centigrade
DNA	-	Deoxyribonucleic acid
Ent	-	Enterotoxin
ELISA	-	Enzyme linked immunosorbant assay
gm	-	Gram
H_2O_2	-	Hydrogen peroxide
HCL	-	Hydrogen chloride
hrs	-	Hours
Kabab	-	Meat cutlet
Khoa	-	Milk concentrate used for the preparation of sweets

KI	-	Potassium iodide
KOH	-	Potassium hydroxide
M	-	Molar
mec A	-	Methicillin resistant gene A
Mec A	-	Methicillin resistant gene A
$MgCl_2$	-	Magnesium chloride
µg	-	Microgram
µl	-	Microliter
ml	-	Milliliter
mM	-	Millimole
mm	-	Millimeter
MRSA	-	Methicillin resistant *Staphylococcus aureus*
MSSA	-	Methicillin sensitive *Staphylococcus aureus*
NaCl	-	Sodium chloride
no.	-	Number
nuc	-	Nuclease gene
NS	-	Normal saline
Paneer	-	Cottage cheese
PBPs	-	Penicillin binding proteins
PCR	-	Polymerase chain reaction
%	-	Percentage
pmol	-	Picomole

PFGE	-	Pulse field gel electrophoresis
PPA	-	Phenolphthalein diphosphate agar
REA	-	Restriction endonuclease analysis
RFLP	-	Restriction fragment length polymorphism
RTD	-	Routine test dilution
S. aureus	-	*Staphylococcus aureus*
Sage	-	Superantigens
SEs	-	Staphylococcal enterotoxins
se	-	Staphylococcal enterotoxin gene
SG	-	Specific gravity
TSB	-	Tryptone Soya Broth
TSST-1	-	Toxic Shock Syndrome Toxin-1
U	-	Unit
VISA	-	vancomycin – intermediate *Staphylococcus aureus*
VRSA	-	vancomycin – resistant *Staphylococcus aureus*

INTRODUCTION

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1. INTRODUCTION

Staphylococcus aureus is a well known pathogen of man and animals (Sandel *et al.*, 2003; Cucarella *et al.*, 2004; Salasia *et al.*, 2004; Dar *et al.*, 2006; and Orrett, 2008). Despite the development of antimicrobial agents the staphylococcal infection still remains an important cause of morbidity and mortality (Mathur *et al.*, 2000; Coast *et al.*, 2003; Kim *et al.*, 2003; Sabour *et al.*, 2004; and Haveri *et al.*, 2008). The spectrum of disease produced by this organism varies from pyogenic infections to toxin-mediated phenomenon (Shanson, 1981). It also causes serious infections in man including deep-seated abscesses, bacteraemia, endocarditis, osteomyelitis, food poisoning etc. (Brakstad, 1992). The main reservoirs of infection are infected cases and carriers who spread infection in a hospital or in a community through droplets in air or through fomites. Carriage in nose plays an important role in its epidemiology and pathogenesis (Lowy, 2002).

In animals too it causes a wide spectrum of diseases like intramammary infections in lactating animals (Waage *et al.*, 1999; and Cucarella *et al.*, 2004) sporadic septicaemia in pigs, abscesses, synovitis, arthritis, osteomyelitis, dermatitis in poultry and ducks (Jones *et al.*, 1990). Bovine mastitis is one of the most important bacterial diseases in dairy cattle throughout the world, and it is responsible for great economical losses to milk producers as well as to the milk processing industries (Martin *et al.*, 2003 and Emery *et al.*, 2006) and also poses antimicrobial resistance threats (Haveri *et al.*, 2008; and Defra, 2008) in veterinary medicine. The ability of *Staphylococci* to produce coagulase, an enzyme that clots plasma was first reported by Loeb in 1903. However, there is no convincing evidence that coagulase is

directly involved in pathogenicity. It has been proposed that the coagulase may inhibit phagocytosis and protect the cocci from antibacterial substances in tissue fluids by laying down a fibrin barrier around them and walling off the lesions. Its production is the principal criterion used by the clinical microbiologists for the identification of *S. aureus* isolates from human infection.

A novel typing method for *Staphylococcus aureus* based on polymerase chain reaction amplification of the variable region of the coagulase gene followed by *AluI* restriction fragment length polymorphism (RFLP) was developed (Goh *et al.*, 1992).

S. aureus strains produce an extra cellular "Thermostable nuclease" (Tnase) a protein with a molecular mass of 17,000 KDa and is considered as a virulence marker. The *nuc* gene is widely employed as the target gene for specific detection of *S. aureus* (Wilson *et al.*, 1991; Kim *et al.*, 2001; Tamarapu *et al.*, 2001; and Ramesh *et al.*, 2002). Jasper (1973) found a close correlation between thermostable nuclease and coagulase production. PCR has the potential for the rapid diagnosis of *Staphylococcous aureus* infection.

Balban *et al.*, (2000) reported that food borne disease has a major impact on public health. In a study of food poisoning in England, the most prevalent contaminated foods were ham (75%), poultry or their products, other contaminated food products including fish and shellfish (7%) and milk products (8%). Contamination occurred most often at homes followed by restaurants and food stores (Wieneke *et al.*, 1993). The milk and dairy products are probably the types of foods most frequently implicated in food poisoning outbreaks (Zehren and Zehreen, 1968; Troller, 1976; DeBuysen *et al.*, 1985;

Genigeorgis, 1989; Leploute, 1994; Adesiyun *et al.*, 1998; and Asao *et al.*, 2003). A variety of exoproteins produced by *S. aureus* cause disease in the mammalian host (Salasia *et al.*, 2004). The most notable virulence factors associated with *S. aureus* are enterotoxins (Dinges *et al.*, 2000). Contamination of food with *S aureus* during storage could lead to the production of enterotoxin. This intoxication resulting from the ingestion of food containing preformed heat stable enterotoxins results in acute disease known as Staphylococcal food poisoning (Gilmour and Harvey, 1990 and Su and Wong, 1997).

In the past, a number of techniques have been developed for detection of Staphylococcal enterotoxins like immunodiffusion (Casman *et al.*, 1969), ELISA (Notermans, 1983) reverse passive latex agglutination (Shingaki *et al.*, 1981) and immunoblotting (Orden *et al.*, 1992). But disadvantages of these methods are that they are slow, relatively insensitive, and are more expensive. In addition, they may give false positive results due to interference by food components and protein A (Orden *et al.*, 1991). Johnson *et al.*, (1991) reported the PCR procedure, which rapidly and specifically detects genes for *staphylococcal* enterotoxins by using synthetic oligonucleotide primers. Polymerase chain reaction offers the possibility of specific amplification of the genes responsible for the SEs production (Saiki, 1988). The studies on detection of enterotoxins from foods of animal origin and clinical specimens are available worldwide but there is paucity of literature on this aspect in India.

The prognosis of patients with *S. aureus* infections was extremely poor before the advent of antibiotics. In early days of antimicrobial era, most infections caused by *S. aureus* were sensitive to

penicillin and other antibiotics. Subsequently strains acquired resistance to previously effective antimicrobials (Duckworth, 1993). They developed resistance to penicillin by virtue of beta-lactamase production, an enzyme, which destroys penicillin. Penicillin-resistant *S. aureus* strains persist in the environment, in carriers and in paramedical staff in hospitals and community (Klimek, 1976; Peacock *et al.,* 1980; Locksley *et al.,* 1982; www.net doctor co. Uk, 2009). The pathogen causes both nosocomial and community acquired infections. At a time when majority of nosocominal *S. aureus* isolates were already resistant to penicillin, the introduction in 1960 of newer penicillinase resistant semi-synthetic penicillin was seen as a major therapeutic breakthrough. These beta-lactamase resistant penicillins include methicillin, oxacillin, nafcillin, cloxacillin, flucloxacillin and others. Unfortunately, just one year later, the first methicillin resistant *S. aureus* (MRSA) was isolated in London (Jevons, 1961). Since then, there have been many reports of MRSA causing various infections throughout the world (Parker and Hewitt, 1970; Locksley *et al.,* 1982; Rao *et al.,* 1990; and Mathur *et al.,* 1994). This form of resistance encompasses all resistant beta-lactam antibiotics including cephalosporins (Sabath, 1982). Beta-lactamase in *S. aureus* can be either intrinsic or chromosomal and is due to the presence of a distinctive gene *mec* A, which encodes penicillin binding proteins (PBPs).

The emergence of antibiotic resistant organisms and their spread in the community has been a subject of increasing concern (WHO report, 1983). *S. aureus* is one of the commonest causes of hospital-acquired infections and the emergence of methicillin-resistant *Staphylococcus aureus* is one of the worst nosocomial hazards. MRSA

can cause infections like pneumonia, postoperative infections, bacteraemia and other infections in community and referral hospitals. MRSA are often resistant to the penicillinase resistant penicillins, tetracyclines, erythromycin, gentamycin, clindamycin, neomycin and trimethoprim. Clones of multidrug-resistant MRSA have become notorious hospital acquired organism causing serious, even fatal, infections in patients admitted for other diseases. In addition to antibiotic resistance, MRSA is often more readily colonized and transmitted. The outbreaks of nosocomial infections are difficult and expensive to control.

It is difficult to identify factors, which contribute to the persistence of MRSA carriage. Excessive antibiotic usage has been incriminated as one of the factors. The transmission and acquisition of MRSA is a multifactorial process depending not only on organism factors but also on host factors. The reservoirs of MRSA are wounds, intravenous catheters, tracheostomies and sites of dermatitis. Some hospital personnel may become chronic carriers.

The present study was therefore carried out to evaluate the phenotypic and genotypic characters of *S. aureus*, which might provide an understanding of the distributation of prevalent *Staphylococcus aureus* clones in India and might help in the development of steps to control *Staphylococcus aureus* infections in man and animals. Moreover, understanding genetic diversity of the organism may have far reaching implications for public health intervention strategies such as tracking the global spread, and understanding the emergence of drug resistance in *Staphylococcus aureus.*

Aims and Objectives

Following were the aims and objectives of this study:

1. Phenotypic characterization of *Staphylococcus aureus* isolates obtained from clinical and animal-origin food samples.
2. To detect the presence of coagulase, thermostable nuclease and commonly encountered enterotoxin genes in the isolates.
3. Molecular characterization by Coa-PCR-RFLP in clinical and animal-origin isolates.
4. To study any similarity or diversity in isolates obtained from clinical and animal-origin samples.
5. To study the resistance rates and patterns of *Staphylococcus aureus* isolates.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. General description

The genus *Staphylococcus,* includes 32 species; among them *S. aureus* has been identified as one of the most important human pathogen (Sheagren, 1984). The organism successfully adapts itself on the ectodermis of warm blooded animals. Some *staphylococci* are members of normal flora of the body surfaces of man and animals; others cause suppuration, a variety of pyogenic infections, food poisoning etc. *Staphylococcus aureus* causes infections in all age groups. It is responsible for sporadic infections as well as epidemics (Locksley *et al.,* 1982; and Thompson *et al.,* 1982) and is the most common cause of infections in hospitalized patients (Dar *et al.*, 2006).

Staphylococcus aureus mainly spreads by person-to-person contact where the source may be an individual with an open draining lesion or an asymptomatic carrier. It may be transmitted by airborne droplets or contact with contaminated objects.

2.2. Historical background

Von Recklinghausen (1871) first observed staphylococci in pus obtained from human pyogenic lesions Further Robert Koch (1878) reported the presence of small spherical bacteria in pus, abscess and in infected blood of people suffering from pyaemia and named them as "*Micrococci*". Later in 1881, a Scottish surgeon Sir Alexander Ogston established the functional role of cocci in abscess and other suppurative lesions. In 1882, these cocci were named as *Staphylococcus*. The first person to isolate pure culture of *staphylococci* in laboratory was Rosenbach (1884). He gave the typical characteristic features of this organism and formal description of the genus. He divided staphylococci

into two species namely *S. aureus* and *S. albus* on the basis of their orange and white-pigmented colonies, respectively. Third species known as *S. citreus*, was added by Passet (1885) based on their lemon yellow pigmentation.

In 1963, Baird and Parker classified the Gram-positive, catalase positive cocci into three main groups; group I- *Staphylococcus*, group II- *Micrococcus* and group III- *Sarcina*. Depending upon the production of enzyme coagulase, *staphylococci* were subdivided as subgroup I - which produced coagulase enzyme were designated as coagulase positive *staphylococci* (*S. aureus*, *S. intermedius*) and subgroups II- VI which do not produce coagulase were designated as coagulase negative *staphylococci*. Members of genera *Staphylococcus* and *Micrococcus* have been placed with *Stomatococcus* in the family "*Micrococcaceae*" (Privot, 1961).

Genetic surveys of Gram-positive, catalase positive cocci indicated that *Staphylococcus aureus* have a G + C content of DNA of 30-39 mol%, whereas *micrococci* have a G + C content of 63-73 mol % (Silvestri and Hill, 1965). *Staphylococcus* and *Micrococcus* have been found to differ on the basis of DNA compositions, O-F test, cell wall composition (Schleifer and Kandler, 1972), cytochromes, menaquinones, cellular fatty acids, polar lipids, DNA-rRNA hybridization and comparative oligonucleotide cataloguing of 16S rRNA (Ludwig *et al.*, 1981).

Staphylococcus aureus are Gram-positive cocci measuring 1.0 micrometer in diameter that occur singly, in pairs, tetrads, short chains and irregular grape like clusters. They are nonmotile and nonspore

forming, catalase positive, facultative anaerobes growing better under aerobic conditions. Most species grow in the presence of 10% sodium chloride and at 18-40^0 C. They are susceptibile to furazolidone (100 μg) and resistant to low level of bacitracin (0.04 unit). They are susceptible to lysis by lysostaphin and are relatively resistant to lysis by lysozyme.

2.3. Habitat

The largest population of *Staphylococcus aureus* in the human body is found on the skin, mainly in the axillae, inguinal and perineal areas; and in the anterior nares (Kloos *et al.*, 1991; and Kloos and Bannerman, 1994). Colonization by *S. aureus* may be found in 6% to 24% of newborns after 3-4 days in a well baby nursery (Gooch *et al.*, 1978). Among healthy adults, carrier rates of 11 % to 32 % were detected in the general population of the United States (Millian, 1960).

The largest populations of cutaneous *staphylococci* are found in regions of the skin supplied with large numbers of pilosebaceous units and sweat glands on the skin and mucous membranes surrounding openings to the body surface. *Staphylococcal* populations living on human skin have been observed in the follicular canals, opening to sweat glands, the cepacious lumen of sebaceous follicles, and on the surface and beneath the desquamating epithileal scales (Noble and Somerville, 1974; and Noble and Pitcher, 1978).

Nasal carriage rates of *S. aureus* range from 10% to 40% in normal adult population residing in the community. The organism is also found in the inguinal and perineal area.

Staphylococci have also been found in the pharynx, conjunctiva, mouth, blood, mammary glands, faeces, body discharges, excretions and intestinal, genitourinary and respiratory tracts of their hosts (Elek, 1959). The occurrence of contaminating *staphylococci* in various food products has been studied extensively (Bergdoll, 1989; Genigeorgis, 1989; Mossel and VanNetten, 1990; and Kloos and Lachica *et al.,* 1994). The presence of enterotoxogenic strains of *S. aureus* in food stuff is regarded as a public health hazard because of the ability of these strains to produce intoxication or food poisoning.

2.4. Cell morphology

Staphylococcus aureus is uniformly gram positive in young (18-24 hours) cultures, nonmotile, nonflagellated, nonspore forming bacteria. It appears spherical with an average diameter of 0.5-1.5 μm. The size varies from strain to strain and influenced by the age of cultures and by the medium on which it is grown. The cells of old cultures (>48 hours) are often Gram-variable to nearly Gram-negative. *S. aureus* mainly produces irregular clusters of cells. Organism divides in more then one plane to form irregular clusters.

Encapsulated cells (form mucoid, glistening colonies) are surrounded by a capsule layer (>200 nm thickness) outside the cell wall (Wilkinson, 1983). *Staphylococci* may also have capsules that are thinner than 200 nm (microcapsules), which are not visible by light microscopy or staining by India ink method. Incidence of *S. aureus* strains exhibiting microcapsules is 80-90% (Hochkeppel *et al.,* 1987).

2.4.1. Cell surface components

2.4.1.1. Cell membrane: It consists of lipid - protein bilayer, composed mainly of phospholipids and protein. It represents a selective barrier and fulfils many functions, including electron transport, active transport, participation in septum formation and segregation of DNA. Phospholpids, gloycolipids, menaquinones and caroteroids make up the major lipid components of the membrane.

The pigments of staphylococci which constitute the lipid component of cell membrane are triterpenoid carotenoids having a C_{30} chain (Marshall and Wilmoth, 1981a). In *S. aureus,* carotenoids range from colorless polyisoprene compounds such as squalence, diapophytoene and diaplophytofluence to the yellow, orange and red triterpenoid carotenoids. The main pigment is staphyloxanthin, a compound in which glucose is esterified.

Various proteins have been isolated from the membrane of *S. aureus* such as adenosine triphosphatase, polyprenolphosphokinase (Sandermann and Strominger, 1972), various oxidases and dehydrogenases (Theodore and Weinbach, 1974) and several penicillin binding proteins (PBPs), which catalyze terminal reactions of peptidoglycan biosynthesis (Hayes *et al.,* 1981; and Hartman and Tomasz, 1984).

2.4.1.2. Cell wall: Peptidoglycan and teichoic acid are the major components of staphylococal cell wall. The peptidoglycan amounts to about 50-60% of the dry weigtht (Schleifer and Kandler, 1972) and teichoic acid amounts to about 30-50% of dry weight and is covalently linked to peptidoglycan. The peptidoglycan is the main structural polymer

in the wall and it plays an important role in maintaining the spherical shape of the cell. It is a heteropolymer consisting of glycan chains that are crossed-linked by short peptides. The glycan moiety is made up of alternating β-1, 4 linked limits of N-acetylglucosamine and N-acetyl muramic acid. Peptidoglycan antibodies can be found in normal human and animal sera. The Biological activities of peptidoglycans include endotoxin like properties (Pyrogenicity, complement activation and generation of chemotactive factors), inflammatory skin reaction, inhibition of leucocyte migration, adjuvant activity, mitogenic activity and induction of immunosuppresive cells.

The cell wall teichoic acids are unique ribitol (five carbon monosaccharide) phosphate polymers. Teichoic acid functions in the specific adherence of Gram-positive bacteria to mucosal surfaces. Precipitating antibodies to teichoic acids can be found in sera of patients with staphylococcal infections (Daugharty *et al.*, 1967; and Crowder and White, 1972).

2.4.1.3. Protein A (SPA): It is a group specific antigen found in the cell wall of about 90% strains of *S. aureus* (especially Cowan 1 strain). Protein A is bound to the cell wall peptidoglycan and it is also shed into the medium during growth of the bacteria. In *S. aureus* strains A676 and in many methicillin-resistant strains, SPA is an extra cellular protein excreted into the culture supernatant.

2.4.1.4. Cell surface adhesins: *Staphylococci* have a group of cell surface proteins that act as adhesins (also known as microbial surface components recognizing adhesive matrix molecules) and bind to extra cellular matrix proteins such as fibronectin, fibrinogen, laminin, elastin and collagen etc. of their host. These proteins bind to eukaryotic host

cells via cell surface receptor proteins (integrins). The adhesins act as targeting factors, attaching bacteria to certain host tissues via matrix proteins. This is an early step in the process of pathogenesis, leading to colonization and subsequent invasion of host cells (Lindberg *et al.*, 1990).

2.4.1.5. Capsules: It is located external to cell wall and is mainly composed of antigenic polysaccharides (Sutherland, 1977). The capsular polysaccharides are made up of different combination of either aminouronic acids or non–aminouronic acids and N-acetyl-D-furosamine or galactose dependent on particular strain (Wilkinson, 1983). *S. aureus* strains can be assigned to at least 11 capsular serotypes based on capsular polysaccharides immunotyping. Of these serotypes 5 and 8 are associated with microcapsules and are the one of most frequently encountered in bactereamia and a variety of other infections (Hochkeppel *et al.,* 1987).

2.4.1.6. Slime: Is a complex extra cellular substances produced in varying amount by many *Staphylococci.* Crude slime is a very heterogeneous substance, usually composed of a variety of monosaccharides, including mannose, galactose, glucose, glucosamine and glucouronic acid, proteins and small peptides.

2.4.2. Extra- cellular protenis

The extra cellular proteins produced by Staphylococcus can act as either toxins or as enzyme activator or non-toxic enzymes. These are as follows:

2.4.2.1. Toxins

2.4.2.1.1. Membrane damaging toxins: Damage the plasma membranes of eukaryotric cells (Thelestam, 1983).

(a) Hemolytic toxins (Hemolysirs): Attack the membranes of erythrocytes, as well as some other cells, from various host species, *S. aureus* produces at least 24 types of hemolysins known as α, β, γ and δ-toxins. A high percentage (86-95%) of human *S. aureus* strains produce α-toxin. γ-toxin is produced by many strains of *S. aureus*. δ-toxin, also known as δ-hemolysin, is one of many virulence factors produced by *S. aureus*. The best characterized and most potent membrane-damaging toxin of *S. aureus* s α -toxin. It is expressed as a monomer that binds to the membrane of susceptible cells. Subunits then oligomerize to form heptameric rings with a central pore through which cellular contents leak. α-toxin is a single polypeptide chain having a molecular weight of 33,000 daltons and an isoelectronic point of 8.5. α-toxin is also known as α –hemolysin.

In humans, platelets and monocytes are particularly sensitive to α -toxin. Susceptible cells have a specific receptor for α -toxin which allows the toxin to bind causing small pores through which monovalent cations can pass. The mode of action of alpha hemolysin is likely by osmotic lysis. This toxin has been shown to be lethal in animals, causing respiratory paralysis, vascular and smooth muscles spasms and tissue necrosis.

(b) Leucocidins: act on leucocytes (granulocytes and macrophages).

2.4.2.1.2. Pyrogenic exo-toxins: Superantigens cause fever and shock in their hosts. These toxins are classified as superantigens (SAgs) and

include the enterotoxins and TSST-1 (toxin shock syndrome toxin-1). Enterotoxin F is also known as toxic shock syndrome toxin (TSST-1). Superantigens are potent activators of T lymphocytes resulting in the liberation of cytokines such as tumor necrosis factor and they bind with high affinity to mononuclear cells. These characteristics partly explain the florid and multisystem nature of the clinical conditions associated with these toxins.

2.4.2.1.3. Enterotoxins: The most notable virulence factors associated with *S. aureus* food poisoning are enterotoxins (Baleban *et al.*, 2000; Joblonski and Bohanch, 2001; Jones *et al.*, 2002; Asao *et al.*, 2003; Lelair *et al.*, 2003; and Alarcon *et al.*, 2005). Enterotoxins are perhaps the best recognized causative agents of food poisoning; although their role in other diseases is being actively investigated (Bergodoll, 1983).

The first report that associated *staphylococci* with food poisoning was probably by Vaughan and Sternberg, in 1884 described an investigation of a large outbreak of illness in Michigan believed to have been caused by eating cheese that was contaminated with *staphylococci.* The genetics of *S. aureus* enterotoxin production have been well studies. The staphylococcal enterotoxin genes are carried either by plasmids (SED and SEJ) (Bayles and Landolo, 1989; and Zhang *et al.*, 1998) by phages (SEA and SEE) (Betley and Mekalanos, 1985), by chromosomes (SEB, SEC, SEG, SHE, SEI, SEK, SEL, SEM, SEN, SEO, SEP and SEQ). Staphylococcal food poisoning (enterotoxicosis) is classified as a disease of moderate severity and limited spread (Anon, 1985). *S. aureus* can readily multiply in many food items, but milk and dairy products are probably the types of foods most frequently implicated (Genigeorgis, 1989; Lepoutre, 1994; Adesiyun *et al.*, 1998; and Asao *et al.*, 2003). Raw

milk and cheese have been repeatedly involved in staphylococcal food poisoning outbreaks (Zehren and Zehreen, 1968; Troller, 1976; and De Buyser *et al.,* 1985). The SEC is an important cause of food borne intoxication and is commonly produced by *S. aureus* isolated from dairy cattle (Wilson *et al.*, 1991; and McLauchlin *et al.*. 2000).

Heat treatment even at 60^0C for 30 minutes could not inactivate SEC (Berja *et al.*, 1967). Untreated mastitis is one of the main causes of continuous economical losses in the dairy industry. The presence of *S. aureus* in the food can be a potential risk for health, particularly if the food is not stored at the prescribed temperature (Thacikova *et al.,* 2003).

Over the last few years PCR assay has been developed to identify specific sequences for SEs by DNA amplification (Johnson *et al.*,1991; Mullis and Erlich, 1998; and Saiki *et al.*, 1998).

DNA amplification method (Polymerase Chain Reaction) can show the presence of enterotoxogenic strains of *S. aureus* before the expression of enterotoxins on the basis of specific gene sequences and in this way can detect the potential source of contamination. The advantage of the PCR method is that it is able to detect genes, for the production of staphylococcal enterotoxins even after heat treatment of food because the DNA remains unchanged (Tsen and Chen, 1992; and Holeckova *et al.*, 2000). *S. aureus* is a pathogen that ranks in top three causes of food poisoning outbreaks in Taiwan (Wang *et al.*, 1983). *S. aureus* is also the third most common cause of confirmed bacterial food borne disease in United States (Bean *et al.*, 1996).

2.4.2.1.4. Epidermolytic toxins: The most dramatic manifestation of epidermolytic toxin is the scalded skin syndrome where the toxin spreads systemically in individuals who lack neutralizing antitoxin: extensive

areas of skin are affected which, after the development of a painful rash, slough off; the skin surface resembles scalding. Such blistering lesions are seen mainly, but not exclusively, in small children. *S. aureus* mediated production of exfoliative toxin also results in a spectrum of blistering skin disorders ranging from localized bullous impetigo to a severe generalized form, scalded skin syndrome. The syndrome is a generalized exanthematous disease consisting of cutaneous tenderness and widespread superficial blistering and denudation. Exfoliative toxins (ETs) are made by certain strains of *S. aureus* (usually phage group 2). Exfoliatin A and B (ETA and ETB) are two serologically distinct proteins produced by *S. aureus*. ETs are serine proteases that bind to the cell adhesion molecule desmoglein 1 and cleave it, resulting in a loss of cell-cell adhesion. Consistent with the expression pattern of desmoglein 1, which is expressed in the upper part of the epidermis, the epidermolysis takes place usually between the stratum spinosum and granulosum. This results in a very thin-walled, flaccid blister that is easily disrupted, exhibiting a positive Nikolsky sign. The pathophysiology of ET resembles that of the autoimmune blistering disease pemphigus.

2.4.2.1.5. Fibrin forming and fibrinolytic enzymes

(a) Staphylocoagulase: Is a protein that clots plasma in the absence of Ca^{++} but requires a coagulase reacting factor (CRF). CRF reacts with coagulase and resulting coagulase–CRFcomplex (staphylothrombin) converts fibrinogen to fibrin (Drummond and Tager 1963). 98–99% of *S. aureus* strains exhibit coagulase activity. Coagulase activity may also be exhibited by the species *S. intermedius*, *S. hyicus*, *S. delphini* and *S. schleiferi* subsp. coagulans.

(b) Staphylokinase: Is a protein that exhibits fibrinolytic activity indirectly by binding to plasminogen, is produced by a relatively high percentage (60-95%) of human *S. aureus* strains of biotype A.

2.4.2.1.6. Bacteriolytic enzymes: Two types of bacteriolytic enzymes are produced by it, glucosaminidase and lysostaphin. The former is produced by *S. aureus* and is active against *Bacillus* and *Micrococcus* sp. Lysostaphin is produced by *S. simulans* biovar *staphylolyticus*.

2.4.2.1.7. Hydrolytic enzymes: *S. aureus* produces lipases, esterases, nucleases, urease, hyaluronidase and endopeptidase.

2.5. Culture characteristics of *Staphylococcus aureus*

They are aerobes and facultative anaerobes. Optimum temperature for growth is 37^0 C (ranging being 12-44^0 C) and optimum pH is 7.5. They can grow well on a variety of selective and non-selective media.

2.5.1. On Nutrient agar

After overnight incubation at 37^0 C, it forms colonies 1-3 mm in diameter, smooth, circular, entire, slightly raised (low convex), glistening, densely opaque with a butyrous consistency. Older colonies become translucent and sticky. Most strains produce golden yellow pigment. Pigment production occurs optimally at 22^0 C and only in aerobic cultures. Pigmentation is enhanced on fatty media such as tween 80 agar, by prolonged incubation and by leaving plates at room temperatures, 1% glycerol monoacetate agar, and milk agar Pigment is not formed in liquid media. Nonpigmented strains are not uncommon. These grow anaerobically and are often smaller and grayish in color.

2.5.2. On Blood agar

Colonies are similar to those on nutrient agar, but may be surrounded by a zone of β- haemolysis, especially when the medium contains 5% sheep, ox, human or rabbi blood and is incubated in an atmosphere of 20% CO_2.

2.5.3. On MacConkey agar

Colonies are smaller and are pink due to lactose fermentation.

2.5.4. On Mannitol salt agar

Yellow colonies 1mm in diameter are formed surrounded by yellow zones due to acid formation.

2.5.5. On Baird- Parker agar

Black colonies surrounded by opaque zone due to lecithinase activity.

2.5.6. On other commercial media

They grow well in a variety of commercially prepared liquid media including TSB, BHI, and nutrient broth, tryptose phosphate broth with or without addition of blood.

2.6. Biochemical properties

S. aureus ferments glucose, maltose, lactose, sucrose and mannitol with production of acid but no gas. Anaerobic utilization of mannitol is considered to be distinctive features of *S. aureus*. It is catalase positive, oxidase negative, methyl red and Voges-Proskauer test positive. Nitrate is reduced to nitrite and indole is not produced. *S. aureus* produces deoxyribonuclease (DNase), and heat stable nuclease

(Thermostablenuclease, TNase). *S. aureus* also produce phosphatase. *S. aureus* is sensitive to novobiocin but resistant to bacitracin.

2.7. Resistance to physical and chemical agents

Staphylococci survive well in the environment under both moist and dry conditions and in laboratory cultures such as sealed agar slopes. It withstands moist heat at 60^0 C for 30 minutes. It is readily killed by phenol and hypochlorite disinfectants at standard in use concentrations and by antiseptic preprations such as hexachlorophane, chlorhexidine and providone- iodine.

2.8. Pathogenecity

2.8.1. Pathogenecity of *Staphylococcus aureus*:

The Staphylococci are normal inhabitants of the body surface of man and animals. The ability to cause disease is their secondary feature. The most important pathogen in the genus is *Staphylococcus aureus* that causes infections and epidemics (Locksley *et al.*, 1982; Thompson *et al.*, 1982; Kim *et al.*, 2001 and Warren, 2005). It is the most common cause of infections in hospitalized patients (Orrett, 2002).

S. aureus is transmitted mainly by person to person contact with the source being an individual with an open draining lesion or an asymptomatic carrier. Less frequently, it may be transmitted by airborne particles or contact with contaminated objects.

2.8.1.2 Pathogenesis of Infections

The five stages in the pathogenesis of *S. aureus* infection are (i) colonization (ii) local infection (iii) systemic dissemination and / or sepsis (iv) metastatic infection and (v) toxinosis (Archer, 1998).

Approximately 35% of healthy individuals are colonized by *S. aureus*, usually in the anterior nares, but also in the throat (4 to 64 %), vagina and perianal area. The organism can be carried asymptomatically for weeks or months on mucous membranes but is only transiently carried on intact skin.

The infection can spread locally (eg. carbuncles, cellulitis, and impetigo or wound infection) or gain access to blood. Once in the blood, the organism spreads widely to peripheral sites in distant organs and septic shock can ensue. As a result of hematogenous dissemination, a number of specific staphylococcal infections can result (eg. endocarditis, osteomyelitis, renal carbuncle, septic arthritis, or epidural abscess). Finally if the organism itself does not invade the blood stream, specific syndromes can results from the local or systemic effects of specific toxins (eg. toxic shock syndrome, scalded skin syndrome, and food borne gasteroenteritis).

Certain underlying conditions or behaviors can predispose individuals to staphylococcal infections, including diabetes mellitus (Cluff *et al.*, 1965), indwelling intravenous drug abuse (Mathew *et al.*, 1995) and AIDS (Jacobson *et al.*, 1988), chronic skin disease like atopic dermatitis. The success of the organism as a pathogen and its ability to cause a wide range of infections are due to its extensive virulence factors.

Virulence factors of *Staphylococcus aureus* and their proposed pathogenic activities.

Virulence factor	Activity
Exoproteins	
α-Lysin	Formation of biofilm, causing respiratory paralysis, Vascular and smooth muscles spasm and tissue Necrosis, osmotic lysis in animals
β-Lysin	Impairment of membrane
λ-Lysin	permeability;
δ-Lysin	cytotoxic effects on phagocytic
panton-Valentine leucocidin	and tissue cells
Epidermoltyic toxins	spectrum of blistering skin disorders ranging from localized bullous impetigo to a severe generalized
ETA and ETB	form, scalded skin syndrome. Cutaneou and tenderness and widespread superficial denudation.Cause blistering of skin
Toxic shock syndrome toxin	Induces multisystem effects; superantigen effects
Enterotoxins	Induce vomiting and diarrhoea; superantigen effects
Coagulase	Converts fibrinogen to fibrin in plasma
Staphylokinase	Degrades fibrir
Lipase	Degrades lipid
Deoxyribonuclase	Degrades DNA
Cell surface proteins	
Protein A	Reacts with Fc region of IgG
Clumping factor	Binds to fibrionogen
Fibronecting-binding protein	Bind to fibronectin
Cell wall polymers	
Peptidoglycan	Inhibits inflammatory response; endotoxin-like activity
Teichoic acid	Phage adsorption; reservoir of bound divalent cations

There are also specific global regulatory systems, such as agr and sar, that determine which virulence factors are produced at specific times during growth and in response to the local environment (Projan *et al.*, 1997).

2.8.1.3. Spectrum of infection

Infections or syndromes for which *S. aureus* is a prominent pathogen.

(a) Primary infections:

Superficial infections

- Folliculitis
- Furunculosis or carbuncle
- Impetigo bullosa
- Cellulitis

Deep infections

- Surgical wound infections
- Epidural abscess
- Osteomyelitis
- Pneumonia
- Empyema

(b) Secondary Infections:

- Eczema
- Decubitus ulcers

- Atopic Dermatitis

(c) Generalized Infections:

- Septicaemia
- Hospital acquired bacteremia
- Acute endocarditis
- Septic arthritis
- Botriomycosis
- Renal carbuncle
- Meningitis

(d) Toxin -mediated diseases:

- Food poisoning
- Toxic shock syndrome
- Scalded skin syndrome

2.9. Staphylococcal antimicrobial resistance

Antibiotic era started in early 1940. with the discovery of penicillin in 1929 by Fleming and the conclusive demonstration of usefulness in infection by Florey and Floery in the early 1940s. In 1944 to 1945, resistance rate of 12% to 22% was documented for *S. aureus* clinical isolates (Bondi, 1945; and Gallerdo E., 1945). The introduction of the semisynthetic β-lactamase-resistant penicillin, such as methicillin and oxacillin brought about a general decline in the prevalence of multiple resistant *S. aureus* during the early 1960s (Shanson, 1981; Orrett *et al.*,

2006). Although resistance to methicillir was detected in approximately 1% of isolates from the United Kingdom, such strains did not pose a serious threat to the overall effectiveness of the antibiotic (Parker *et al.*, 1974). By the late 1960s to early 1970s, strains resistant to the semi-synthetic β-lactams were isolated with increasing frequency in a number of countries including Australia (Rountree *et al.*, 1968; and Rountree *et al.*, 1973), Belgium (Klastersky *et al.*, 971), France (Courtieu *et al.*, 1964), Poland (Borowski *et al.*, 1964), the United Kingdom (Parker *et al.*, 1974), and the United States (Barrett *et al.*, 1968; and O, Toole *et al.*, 1970), Denmark (Bulow, 1971) and Switzerland (Kayser, 1975; and Kayser *et al.*, 1972). These strains formed a significant proportion of the *S. aureus* isolates. Most methicillin-resistant strains isolated at this time produced a β-lactamase and were also resistant to streptomycin, sulfonamides, and tetracycline; many demonstrated additional resistance to chloramphenicol, erythromycin, and fusidic acid or to the aminoglycoside, neomycin (Lacey, 1975 and Orrett, 2006).

Dermerec obtained first evidence of resistance to penicillin in *S. aureus* in 1945. Several studies conducted later on confirmed genetic origin of drug resistance to penicillin (Barber, 1948; Rountree and Jhonson, 1949; Lederberg and Lederberg, 1952; Altermeier and Gulbertson Vetto, 1955).

Prevalence of penicillin resistant clinical isolates increased rapidly at Boston city hospital, Boston. A minimal inhibitory concentration (MIC) of greater than 0.04 microgram of penicillin per ml was found in 17% of *S. aureus* isolates prior to 1946; 75% of isolates in 1946 to 1949; 79% in 1948 to 1949. An MIC of greater than 25 microgram / ml was found in 28 % of the isolates in 1946 to 1947, and 73% in 1951. At present,

approximately 5% of *S. aureus* isolates still are susceptible to penicillin (Parvez *et al.*, 2004). Penicillin resistant *S. aureus* from US hospital obtained in 1971 to 1982 revealed a 17% decrease in susceptibility with about 90% isolates resistant to penicillin in 1982. High percentages of penicillin resistant *S. aureus* were reported by various workers (Kosmidis *et al.*, (1988) 80%; Vijayalakshmi *et al.*, (1980), 98.6%; and Refsahl *et al.*, (1992), 80.9%; Tambekar *et al.*, (2007), 100%). During subsequent years after discovery of penicillin, other potent antibiotics like neomycin, streptomycin, tetracycline, chloramphenicol, macrolides and other newer antibiotics were discovered. Staphylococci quickly developed resistance to these agents. Tetracycline was increasingly used during the 1950s to treat many different infections in hospitals, including penicillin resistant *S. aureus* infection. The first tetracycline resistant staphylococci appeared within a few years of the introduction of tetracycline. The increased incidence of tetracycline resistance was almost certainly due to hospital cross infection by *S aureus* strains containing a plasmid for tetracycline resistance. Erythromycin was widely used to treat infections due to penicillin and tetracycline resistant *S. aureus* soon after it was introduced in 1952. Resistance to erythromycin in staphylococci appeared during 7-10 days of erythromycin treatment of patients with staphylococcal infection.

In some hospitals erythromycin resistant staphylococci with penicillin tetracycline erythromycin (PTE) or penicillin streptomycin tetracycline erythromycin (PSTE) patterns of resistance emerged and spread rapidly while in others erythromycin resistance became a problem only after many months (Lowburg, 1960). Cross resistance to other macrolides, including oleandomycin and spiramycin was generally present in strains

resistant to erythromycin. Neomycin-resistant *S. aureus* strains had first been detected in the United States in 1950 to 1960 (Finegold *et al.*, 1960; and Parker *et al.*, 1970) and soon appeared elsewhere (Jacobs *et al.*, 1963; Robertson, 1963; and Rountree *et al.*, 1965). Resistance to neomycin, and to the related aminoglycosides, kanamycin and paromomycin, had emerged after a delay of approximately 10 years and was attributed to the widespread topical use of neomycin on the skin and in the nose (Lowbury *et al.*, 1964; and Rountree *et al.*, 1965). A similar delay occurred between the introduction of the aminoglycoside, gentamicin, in 1964 and outbreaks of hospital infection in 1975 to 1976 caused by *S. aureus* strains concomitantly resistant to gentamicin and to two other aminoglycosides: kanamycin and tobramycin (Bint *et al.*, 1977; Buckwold *et al.*, 1979; McGowan *et al.*, 1979; Speller *et al.*, 1976; Wyatt *et al.*, 1977 and Orrett, 2008). The extensive topical use of the antibiotic was implicated in the appearance and spread of these resistant strains (Noble *et al.*, 1978; Wyatt *et al.* 1977 and ISAAR, 2007). Prior to these outbreaks gentamicin- resistant isolates were rare in France (Soussy *et al.*, 1975), the United Kingdom (Lacey *et al.*, 1968; and Porthouse *et al.*, 1976), and the United States (Pittman *et al.*, 1965). Reports of *S. aureus* strains resistant to both gentamicin and methicillin, along with a wide range of other antibiotics including penicillin, tetracycline, and streptomycin initially came from London, England (Shanson *et al.*, 1976), in 1976, although similar strains were subsequently reported to have been present in Dublin, Republic of Ireland (Cafferkey *et al.*, 1985), and in the United States (Crossley *et al.*, 1979) at that time.

Chloramphenicol was occasionally used as a reserve drug in some hospitals to treat serious infection caused by *S aureus* strains which were resistant to penicillin, streptomycin, tetracycline, and erythromycin (PSTE), during the late 1950s. Koch (1960) reported a substantial increase in the incidence of chloramphenicol resistant staphylococci from 2% of strains in 1953 to 34% in 1958. Cross-resistance among chemically unrelated antibiotics was first noted with chloramphenicol and erythromycin (Barber *et al.*, 1958) and with erythromycin and oleandomycin. In subsequent years, *S. aureus* developed resistance to these antimicrobial agents too. During the late 1970's and early 1980's, strains of *S. aureus* resistant to multiple antibiotics including methicillin and gentamicin were increasingly responsible for outbreaks of hospital infections in countries around the world, e.g. Argentina (Schugurensky *et al.*, 1984), Austria (Spitzy *et al.*, 1979), Australia (Gilbert *et al.*, 1982; King *et al.*, 1982; Pavillard *et al.*, 1982; Pearman *et al.*, 1985; and Townsend *et al.*, 1985), Belgium (Yourassowsky *et al.*, 1981), Denmark (Espersen *et al.*, 1982; and Rosendal *et al.*, 1981), Republic of Ireland (Cafferkey *et al.*, 1985; Cafferkey *et al.*, 1983; and Horne *et al.*, 1981). England (Bradley *et al.*, 1985; Price *et al.*, 1980; and Shanson *et al.*, 1985), France (El Solh *et al.*, 1981; and Goulett *et al.*, 1981), East and West Germany (Naidoo *et al.*, 1983; and Witt *et al.*, 1977), Greece (Giamarellou *et al.*, 1981), Italy (Varaldo *et al.*, 1984), Japan (Konno *et al.*, 1982), and the United States (Craven *et al.*, 1979; Haley *et al.*, 1982; Schaefler *et al.*, 1984; and Wenzel, 1982). Even before methicillin was widely used, a strain of *S. aureus* with natural resistance to this antibiotic was identified by Jevons in 1961 (Jevons, 1961).

Thereafter, methicillin resistant *S. aureus* (MRSA) emerged as a major pathogen worldwide (Colley *et al.*, 1965; Crossely *et al.*, 1979; Fluckiger, 1979; Sares, 1997; Michel *et al.*, 1997; and Ayliffe, 1997). Since the introduction of methicillin into clinical use in 1961, the occurrence of MRSA has steadily increased and hospital borne infections caused by such isolates have become a serious problem worldwide (Hashimoto, 1994; Brown, 2006; Lee, 2001; Kerttula, 2004; Bukhari, 2004; and Orret, 2008). In India, a varying prevalence rate of MRSA (5.9 to 52%) has been reported (Chakravarthy *et al.*, 1988; Udayasankar *et al.*, 1997; Mehndiratta *et al.*, 2001; Vidhani *et al.* 2001; Majumder *et al.*, 2001; Hanumanthappa *et al.*, 2003; Arakere *et al.*, 2005; Dar *et al.*, 2006 and Tiwari *et al.*, 2008).

Mec the structural gene for PBP2', has been located on the chromosomal map of *Staphylococcal aureus* close to the novobiocin resistance gene *nov* (Kuhl *et al.*, 1978). It has been showr to be additional DNA not present in isogenic sensitive st ains (Beck *et al.*, 1986), and is probably transposable (Trees and Lando o, 1983). A specific region of this additional DNA was found to be highly conserved in MRSA and methicillin resistant coagulase negative staphylococci (MRCNS) collected over a 25 year period (Berger-Bächi, 1989; Hartman and Tomaz, 1989; and Murakami and Tomasz, 1989) is involved in the expression of methicillin resistance.

At present no genetic model exists to explain heterogeneous and homogeneous resistance. Low-level methicillin resistance can result from the production of large amount of betalactamase, or increased production and/or modified

penicillin-binding capacity of normal PBPs (McDougal *et al.*, 1986; and Tomasz *et al.*, 1989). Such borderline resistant *Staphylococcus aureus* strains (BORSA) seldom have minimal inhibitory concentrations (MIC) of methicillin exceeding 16 µg/ml, and their clinical significance is through to be limited (Chambers *et al.*, 1996). Song *et al.* (1987) have sequenced *mec* and found considerable homology between the promoter, the initial sequence of *mec* and staphylococcal β – lactamase. Earlier reports that *mec* was carried out on plasmids (Dornbusch *et al.*, 1969) may have been erroneous, or examples of temporary *mec* insertion into the plasmid, as described by Trees and Landolo (1988). *Mec* itself may have within it IS-like elements that may act as a trap for other resistance determinants (Berger-Bächi, 1989).

The prevalence of MRSA increased rapidly worldwide, and glycopeptide antibiotics have been widely relied upon to treat MRSA infections. Glycopeptide antibiotics are a class of antibiotic drugs composed of a glycosylated cyclic or polycyclic nonribosomal peptides. Significant glycopeptide antibiotic include vancomycin, teicoplanin, telavancin, bleomycin, ramoplanin and decaplanin. This class of drugs inhibit the synthesis of cell walls in susceptible microbes by inhibiting peptidoglycan synthesis. The bind to the amino acids within the cell wall preventing the addition of new units to the peptidoglycan. In particular they bind to acyl-D-alanyl-D-alanine in peptidoglycan. They D stands for the dextro stereoisomer of the amino acids which is significant as amino acids are normal L or levo steroisomers. In 1996, the first documented infection caused by *S. aureus* with reduced susceptibility to vancomycin (vancomycin – intermediate *Staphylococcus aureus* (VISA) was reported in Japan (Hiramatsu *et al.*, 1997). Walsh *et al.*, 2002 has reported about

20 cases of VISA infection from several countries. Furthermore, 2 isolates of fully ancomycin-resistant *Staphylococcus aureus* were documented in the United States during 2002 (CDCP, 2002). To date, the resistance to vancomycin has not been reported from India (Salaria *et al.*, 2001; Arakere *et al.*, 2005; Shittu *et al.*, 2006; and Dar *et al.*, 2006). Teicoplanin is a newer antibiotic chemically related to vancomycin. Liu *et al.* 1996 have used teicoplanin and vancomycin in a randomized trial and have seen that treatment was successful in 17 (85%) of 20 patients who were randomized to teicoplanin group and in 15 (75%) of 20 patients randomized to vancomycin group. In addition nephrotoxicity was significantly higher with vancomycin. It has been also suggested that teicoplanin, given by intramuscular route, has a longer half life and can be given in once daily dosage (Glupcznski *et al.*, 1986). However, there is no report about use of this drug in India (Salaria *et al.*, 2001).

2.10. Typing of *Staphylococcus aureus*: Strain typing is an integral part of epidemiological investigations of nosocomial infections. All typing system can be characterized in terms of typeability, reproducibility, discriminatory power, ease of performance, and ease of interpretation (Arbeit, 1995).

2.10.1. Phenotypic Methods:

Phenotypic methods are those that characterize the products of gene expression in order to differentiate strains. Properties such as biochemical profiles, bacteriophage type, antigens present on the cell's surface and antimicrobial susceptibility profiles etc. are examples of phenotypic properties that can be determined in the laboratory. Because they involve gene expression, these properties all have a tendency to

vary, based on changes in growth conditions, growth phase, and spontaneous mutation.

2.10.1.1. Antimicrobial Susceptibility: Antimicrobial susceptibility has relatively poor discriminatory power because antimicrobial resistance is under tremendous selective pressure in healthcare institutions (Tenover *et. al.,* 1996) and often is associated with mobile genetic element (e.g. transposons and plasmids) (Davies, 1994). Changes in antibiograms also may reflect spontaneous point mutation, such as seen in fluoroquinolones (Nakamura *et al.*, 1989). Thus, isolates that are indistinguishable may manifest different antimicrobial susceptibilities due to acquisition of new genetic material over the time (Locksley *et al.,* 1982) or the loss of plasmid (Mickelsen *et al.*, 1985). Conversely unrelated isolates may have indistinguishable resistance profiles, which may represent acquisition of the same plasmid by multiple species (a "plasmid outbreak").

2.10.1.2. Serotyping: Serotyping, a nonmolecular method, uses a series of antibodies to detect different antigenic determinants on the surface of the bacterial cell. Serotyping is one of the classic strain typing techniques that have been used over the years for epidemiological studies (Oeding, 1978).

2.10.1.3. Bacteriophage typing: The study of bacteriophage typing was first made by an English bacteriologist. The phage typing of *S. aureus* became possible when Fisk (1942a, b) found a readily available source of phages with a narrow spectrum of activity on different strains of this species. The present phage typing system is a direct descendent of that of Wilson and Atkinson (1945) as modified by Williams and Rippon (1952).

These workers showed that staphylococc' could be characterized by lysis using a single phage, but many differ :nt patterns of lysis could be obtained with a set of phages. The dif'erence between these patterns could be used to make fine distinction between *Staphylopcoccus aureus* strains. However the patterns given by strains of common origin were not always same. It was therefore necessary to record the strength of the reactions and to take account of the known range of variability in interpreting the result. Phage typing patt:rn is expressed as a list of the phages by which staphylococci are lysed strongly, eg. 3c/55/71 or 80/81. Phage typing with stronger phages is n)t as reproducible as typing at routine test dilution (RTD). The strengt· of phage for secondary typing should be RTD x 100 as it results in slight reduction in the percentage of typeable culture but increased accuracy.

A basic set of typing phages w·.s established by international agreement in 1953 and has since undergone several modifications (Report 1959, 1963, 1967, 1971, 1975). A number of new phages were introduced, either to characterize the pre-existent strains (e.g. phage 71) or due to a general fall in the typea'>ilty rate which indicated the appearance of new strains either in hospitals or in the general populations (eg. phage 80, 81, 83A, 84, 94, 95 and 96). The sub committee, on phage typing of staphylococcus, of the International Committee on nomenclature of bacteria has recommended methods to be used for propagating the phages, testing of phage filtrates and typing the strains of *Staphyloccous aureus*. The Staphylo occal Reference Laboratory, Colinade, London, acts as the internati>nal center for staphylococcal phage typing. The International center iss·ies to each national laboratory,

once every four years, a fresh basic set of freeze-dried phage preparations, together with corresponding propagating strains (PS). Each time the phages are propagated, they must be tested by a standard procedure before being used in routine typing.

Currently, the basic set for phage typing consists of 23 phages (Report, 1975). Typing phages are subdivided into small numbers of lytic groups, members of which appeared together in typing patterns, and divided staphylococci into corresponding phage groups (Williams and Rippon, 1952). Three main phage groups could be recognized and were subsequently numbered I, II and III. Strains lysed by phage 42D are rarely of human origin and form part of phage group IV. Phages 94 and 96; and certan other phages not included in the basic set, belong to quite distinct lytic group for which the number V has been proposed (Asheshov *et al.,* 1977). Phages 81, 94, 95 and 96 cannot be allocated to any of the lytic groups.

Group I	29, 52, 52A, 79, 80
Group II	3A, 3C, 55, and 71
Group III	6, 42E, 47, 53, 54, 75, 77, 83A, 84, 85
Not allocated	81, 94, 95, 96

The commonest phage group reported in different studies in India is group III (Dutta *et al.,* 1976). The limitations of bacteriophage typing of *S. aureus* has clearly been recognized for many years in the past (Mulligan *et al.*, 1988, Khalifa *et al.,* 1989; Blumberg *et al.*, 1992; Zierdf *et al.*, 1992; and Schlichting *et al.*, 1993).

Many laboratories over the world are concerned with the problems of nontypeable strains particularly when these are resistant to many

antibiotics. In India, the incidence of untypeability remains high. At the National Typing Center Maulana Azad Medical College, New Delhi, during 1981–82, 53.4% of strains remained untypeable with the basic set of phages. To combat the problem of untypeability some methods were proposed.

Heat shock treatment: Dowsett *et al*, (1984) proposed heat shock treatment. In this method untypable strains were subjected to heat shock at 56^0C for 3 minutes prior to application of basic set of phages. This method could type 27.35% of untypeable strains.

Reverse phage typing: Danceret *et al.*, (1988) proposed this method. It was carried out by inducing lysates of the strains by treatment with mitomycin C and inoculating these lysates on lawns of propagating strains and on indicator strains 1030, W57 and 18042. Patterns were read as those strains on which induced lysates produced any degree of lysis. By this method 90.82% of untypeable strains could be typed.

Supplementary phages for MRSA: (Richardson *et al.*, 1988): A large number of phages have been isolated either by Fisk's technique, to isolate new phages by cross culture method or by growing one of the original phages in the presence of a new strain of *S. aureus* and obtaining its variant. These new isolates and their utility in typing a significant number of strains as compared to phages of basic set led to many changes in the international set. Newly isolated phages must be stable in their characteristics and should be readily propagated at least 10,000 routine test dose and their use depends mainly on their typing potentialities.

Richardson and coworkers isolated phages for MRSA strains from different places and evaluated this phage set as an aid to recognition of isolates of Thames EMRSA. In all, 577 isolates of MRSA were included

in the study. Phage carried by all isolations from one country was spotted on to all isolates from that country; 19 phages were propagated to a sufficiently high titre (616–634). After inspecting the lytic spectrum and taking into consideration case of propagation and stability, the phage set was reduced to 9 phages namely 616, 617, 618, 620, 622, 625, 626, 629 and 630. These phages were found to be a useful adjunct to the international set for typing of MRSA. These supplementary phages were used by JE Coia, 1990, with increased typeability. Despite the problems of untypeability, phage typing still remains a cost effective method for epidemiological studies particularly on a large scale.

2.10.2. Molecular typing:

Genotyping methods are those that are based on an analysis of the genetic structure of an organism including polymorphism in DNA restriction patterns based on cleavage of the chromosome by enzymes that cleave the DNA into hundreds of fragments or into 10 to 30 fragments. Genotyping methods are less vulnerable to natural variations, although they can be affected by insertion or deletion of DNA into the chromosome, the gain or loss of extra chromosomal DNA, or random mutations that may create or eliminate restriction endonuclease sites. Although not all molecular techniques are equally effective for typing all organisms, they can be characterized in terms of typeability, reproducibility, discriminatory power, ease of performance and ease of interpretation. Outbreak investigation of *S. aureus* are viewed as short-term events or as cases of local epidemics, and in these settings mainly genotypic methods are able to distinguish clonal spread from related isolates. In addition to tracking outbreaks, genotyping is used to

distinguish between contaminating and infecting isolates and between separate episodes of infection and relapse of disease (Gouby *et al.*, 1994).

Over the last few years, several molecular techniques have emerged as the methods of choice for typing bacterial isolates, namely restriction endonuclease analysis (REA) of plasmid DNA; REA of chromosomal DNA using frequent cutting enzymes and conventional electrophoresis; restriction fragment-length polymorphism (RELP typing) analysis using DNA probes; pulsed-field gel electrophoresis (PFGE) and arbitrarily primed PCR (AP-PCR) and other related nucleic acid amplification based typing methods.

2.10.2.1. REA of Plasmid DNA: Some strains of bacteria contain only a single large plasmid, often in the size range of 100 to 150 kilo bases (kb). Because it is difficult to differentiate plasmids in this size range, especially those that vary by only 10 kb to 15 kb, some investigators have added a restriction endonuclease digest on step to try to increase the discriminatory power of agarose gel electrophoresis (Pfaller *et al.*, 1991). While this can be helpful, in large plasmids this produces many restriction fragments, which can make interpretation difficult, especially when multiple large plasmids are present.

2.10.2.2. Gel Electrophoresis Techniques for Analysis of Chromosomal DNA: There are two methods of typing organisms based on fragment patterns produced by cleaving chromosomal DNA with restriction endonucleases. The first method, often referred to as conventional electrophoresis, uses a restriction enzyme that cuts the chromosome into hundreds of pieces (frequent cutter), followed by standard agarose gel electrophoresis. Larger fragment coalesce at the top

of the gel or do not migrate into the gel. The second method, PFGE, uses enzymes that cut chromosomal DNA infrequently, generating 10 to 30 bands, followed by a novel form of electrophoresis that can separate fragments from 1 kb up to 1,000 kb (1 mega base).

2.10.2.3. Pulsed-field gel electrophoresis (PFGE): Pulsed-field gel electrophoresis was first described in 1984 as a tool for examining the chromosomal DNA of eukaryotic organism (Schwartz *et al.*, 1984). Subsequently, PFGE has proven to be a highly effective molecular typing technique for many different bacterial species (Arbeit *et al.*, 1995; Swaminathan *et al.*, 1993; and Maslow *et al.*, 1993). In this method, the bacterial genome, which typically is 2,000 to 5,000 kb in size, is digested with a restriction fragments ranging from 10 to 800 kb. Essentially all of these fragments can be resolved as a pattern of distinct bands by PFGE, using a specially designed chamber that position the agarose gel between there sets of electrodes that form a hexagon around the gel. Instead of applying an electric current to the gel in a single direction, as is done in conventional electrophoresis, in PFGE, the current is applied first in one direction from one set of electrodes, then shifts to the second set of electrodes for a short period of time (a pulse), and then that shifts to the third set of electrodes. Thus, the electric field that causes the DNA to migrate in the gel is provided in pulses that alternate from three sets of electrodes. This causes the DNA to wiggle through the gel and the back-and forth movement results in the higher level of fragment resolution seen with the technique. In general, PFGE is one of the most reproducible and highly discriminatory typing techniques available and is currently the typing method of choice for many species. The major difficulties associated with PFGE are related to the technical demands of

the procedure and initial cost of the equipment. Preparation of suitable genomic DNA requires 1 to 3 days depending on the organism tested, and the equipment required (including the electrophoresis apparatus and transilluminator).

2.10.2.4. Typing Methods Using PCR: Polymerase chain reaction has been used for several years for the direct detection of many types of infectious agents in clinical sample (Persing, 1993), has been adjusted for use as a typing tool (Welsh *et al.*, 1990; Williams *et al.*, 1990 and Van Belkum, 1994). The hallmark of PCR is the ability to produce literally millions of copies of a particular DNA segment with high fidelity within 3 to 4 hours time. The procedure requires template DNA which may be present in the sample in minute quantities; two oligonucleotide primers, which flank the sequences on the temple DNA to be amplified (thus defending the starting points for DNA polymers activity); and a heat-stable DNA polymerase. Efficient amplification is accomplished readily for templates of less than 2,000 base pairs, although templates as large as 35 kb now can be amplified by using newer polymerases. A typical PCR assay requires double-stranded DNA melted into single strands; an annealing phase, in which the primers bind to the target sequences on the single strands and an extension phase in which DNA synthesis proceeds from the primers along each strand of the temple DNA, thereby generating two new double-stranded copies of the original template. After 30 such cycles, a single initial copy of template DNA theoretically can be amplified to 1 billion copies.

2.10.2.5. Arbitrarily Primed PCR: Arbitrarily primed PCR also referred to as the randomly amplified polymorphic DNA assay is a variation of the PCR technique employing a single short (typically 10

base pairs) primer that is not targeted to amplify any specific bacterial DNA sequence (Welsh *et al.,* 1990). Rather, at low annealing temperatures, the primer will hybridize at multiple random chromosomal locations and initiate DNA synthesis. If one copy of the primer binds to one strand of DNA, and another copy of the primer binds on the opposite strand of DNA but on proximity of the first primer, a DNA fragment will be synthesized and amplification of that fragment will occur. The resulting PCR products will represent a variety of different-sized DNA fragments that are visualized by agarose gel electrophoresis. This approach has remarkable general applicability and has been applied to typing eukaryotic species, as well as many bacterial species (Van Balkum *et al.*, 1994). The reproducibility and discriminatory power of AP-PCR is a subject of active discussion and investigation.

2.10.2.6. PCR-DNA Sequencing: DNA sequencing of hypervariable gene sequence has shown great promise as a typing tool (DuBose *et al.*, 1988 and Dean *et al.*, 1991). While this technique currently is not feasible for most laboratories, the availability of less expensive DNA sequencing machine may make this technique more accessible in the future.

2.10.2.7. Coagulase restriction fragment length polymorphism (COA–RFLP): Molecular typing of staphylococcal isolates can provide useful clonal information of a staphylococcol outbreak. Numerous methods for *S. aureus* typing have been described such as inter–IS 256 spacer length polymorphisms, protein A polymorphism (Frenay *et al.,* 1994), coagulase gene polymorphisms (Goh *et al.*, 1992) ribosome spacer DNA amplicon polymorphism (Kumari *et al.,* 1997), ribotyping, restriction enzyme profiles of plasmid DNA. Among these

methods PFGE has been demonstrated to have advantages in discriminatory power, typeability and reproducibility and has been taken as the "gold standard" for the typing of *S. aureus* even though it is labor intensive and time consuming in compare to other methods. Coagulase gene restriction profile analysis, a PCR–based method is easy to perform and has high levels of specimen typability and reproducibility, and it has been used successfully for the typing of MRSA isolates (Hookey *et al.*, 1998).

Coagulase is produced by all strains of *S. aureus* (Kloos *et al.*, 1986). Its production is the principal criterion used in the clinical microbiology laboratory for the identification of staphylococcal infection in human beings and it is thought to be an important virulence factor. The sizes and DNA restriction endonuclease site polymorphisms at the 3' coding region of the coagulase gene have been utilized in PCR–based restriction fragment length polymorphism (RFLP) analysis of *S. aureus* (Goh *et al.*, 1992; Lawrence *et al.*, 1996; Schwarzkopf *et al.*, 1994; and Tenover *et al.*, 1994).

Shopsin *et al.* (2000), reported that coagulase gene repeat region nucleotide sequences typing is a useful addition to short sequence repeat region sequencing typing for analysis of *S. aureus*, including MRSA strains. Goh *et al.* (1992), Schwarzkopf *et al.* (1994), Hookey *et al.* (1998), Seifert *et al.* (1999). Muehlherr *et al.* (2003), Montesions *et al.* (2002), Wang *et al.* (2003), Cucarella *et al.* (2004), Scherrer *et al.* (2004). Scherrer *et al.* (2004) and Salasia *et al.* (2004) used PCR–RFLP technique for the typing of *S. aureus* strains using AluI enzyme and observed distinct coa-RFLP patterns.

MATERIALS & METHODS

3. MATERIALS AND METHODS

The present study was conducted in the Department of Microbiology, Jawaharlal Nehru Medical College, A.M.U., Aligarh and the Department of Veterinary Public Health, Indian Veterinary Research Institute, Izatnagar, Bareilly during June 2003 to March 2006. The study aimed for evaluation of phenotypic and genotypic characters of *Staphylococcus aureus* isolates obtained from clinical specimens and from foods of animal origin.

3.1. Collection of Samples:

3.1.1. Clinical Specimens:

A total of 3514 human clinical specimens including Pus, Urine, CSF, Throat swab, Ear swab, Conjuctival swab, Cervical swab, Semen, CSF, Aspirated Fluid, Stool and Vomitus were collected from the patients admitted in various wards or from outdoor patients of Jawaharlal Nehru Medical College and hospital, A.M.U., Aligarh. A total of two hundred thirteen raw milk samples were collected as animal origin clinical specimens from Indian Veterinary Research Institute, Izatnagar, Bareilly. The samples were collected in sterile containers / swabs according to standard techniques (Mackie and McCartney. 2000) for the isolation of *Staphylococcus aureus*.

3.1.2. Food Samples:

A total of 1006 food samples were collected according to the methods described by Agarwal *et al.* (2003). The details of the food samples are provided below:

3.1.2.1. Milk products: About 2 gm each of Khoa (Milk concentrate used for the preparation of sweets), Paneer (cottage cheese), Chamcham and Sweets were collected with aseptic precautions in sterile glass containers.

3.1.2.2. Raw meat: Meat sample used in this study were collected aseptically and quickly transported to laboratory under chilled conditions.

3.1.2.3. Cooked meat Products: About 2 gm each of Kabab (a meat cutlet) and Salami were collected in the sterile polybags aseptically.

3.2. Laboratory procedures:

3.2.1. Direct Microscopic examination: Smears were prepared from all the clinical samples on clean sterile glass slides for Gram's staining. The smears were allowed to dry and then fixed by passing through flame and stained by Gram's technique. The smears were examined under oil immersion to look for Gram positive cocci in clusters.

3.2.2. Processing of food samples:

(a) Processing of milk products: The samples were homogenized at room temperature in 10 ml of NS in sterile beakers. The homogenization was done by electrically operated food homogenizer and then used for bacterial isolation.

(b) Processing of raw meat samples: The surface of the collected meat samples were trimmed off with sterile scissors and forceps. From each sample, a small portion was taken and cut into small pieces and then ground in 10 ml NS by electrically operated food homogenizer.

(c) Processing of cooked meat products: The collected meat products were homogenized in 10 ml NS using a homogenizer and them subjected to culture.

3.2.3. Bacterial culture:

All clinical and food samples, were cultured according to standard procedure appropriate to the type of specimen.

Samples were streaked on 5% Sheep blood agar, nutrient agar and Baird-Parker agar medium and incubated for 24-72 hours at 37^0C. The isolates were stocked in agar stabs and stored at 4^0C for further characterization.

3.3. Phenotypic tests for identification / characterization:

Staphylococci were identified as per standard methods (Mackie and McCartney, 2000).

3.3.1. Colony Morphology: On nutrient agar and sheep blood agar:

The circular, smooth, 1-3 mm, low convex, glistening and opaque colonies, which were easily emulsifiable, butyrous in consistency golden yellow/creamy colour, surrounded by zone of β-hemolysis on blood agar were identified as the colonies of *S. aureus*. On Baird-Parker medium distinct black colored colonies were found.

3.3.2. Microscopic Morphology: Smears were prepared from the colonies for Gram's staining to look for the characteristic morphology of Staphylococci.

3.3.3. Other tests:

The Staphylococcal isolates were further characterized on the basis of:

3.3.3.1. Enzymatic Tests:

3.3.3.1.1. Catalase Test:

Requirement: 3% (V/V) Hydrogen peroxide.

Method: A drop of H_2O_2 was placed on a clean glass slide. Using a clean glass rod, small amount of colony to be tested was picked up from nutrient agar plate and immersed into H_2O_2 drop. Production of gas bubbles immediately indicates positive reaction.

Controls:

Catalase positive control: *S. aureus* oxford strain number 6571.

Catalase negative control: Streptococcus species

3.3.3.1.2. Coagulase Test:

Requirements:

Rabbit plasma

Normal Saline (0.85%) or Nutrient broth

Controls:

Coagulase- positive strain (*S. aureus* oxford strain number 6571).

Coagulase- negative Staphylococci (*S. epidermidis*)

a. Slide coagulase test: (William and Harper, 1946): To detect 'bound coagulase' (Clumping factor):

Method: A drop of physiological saline solution was placed on a clean glass slide and with minimum spreading one or two colonies of culture under test was emulsified in it. A control suspension from a known coagulase-positive and negative culture was also made to confirm the reactivity of the plasma. With inoculating wire, a drop of plasma was added at room temperature and mixed gently. The wire was flamed and the procedure was repeated for control suspensions. The appearance of coarse clumps visible to the naked eye within 10 seconds indicates positive reaction. The absence of clumping or any reaction taking more than 10 seconds to develop was considered as negative.

b. Tube Coagulase Test (Modified from Gillespie 1943): To detect 'free coagulase':

Method:

A 1:10 dilution of rabbit plasma was prepared in saline (0.85% NaCl) solution. 0.5ml of the diluted plasma was placed in a small sterile tube. Few colonies of Staphylococci under test were emulsified in nutrient broth to give a dense suspension. Subsequently 0.1 ml of this suspension (about 10^9 cocci) was added to the diluted plasma tube. Similarly control test with known coagulase–positive and coagulase-negative cultures were set up. A tube of unseeded diluted plasma was also included to confirm that it does not coagulate spontaneously. The tubes were incubated at 37^0C in a water-bath and examined for clot formation by tilting the tube through 90^0 at 1, 2 and

4 hrs. and again if still negative, after standing overnight at room temperature.

Test was read positive when the plasma had been converted into a stiff gel when the tube was tilted or inverted. Test in which the plasma remained wholly liquid or showed only a flocculent or ropy precipitate or free flowing was read as negative.

3.3.3.1.3. DNAse Test (Lachica *et al.*, 1971):

Requirements: DNA agar (Oxoid DNAse Agar) with 0.1% toluidine blue obtained from Hi Media.

Method:

Petriplates were prepared by pouring toluidine blue DNA agar. After solidification, 2 mm diameter wells (10-12 wells per plate) were made and agar plugs were removed by aspiration, 0.01 ml each heated sample (15 min. in boiling water bath) of BHI broth culture was added into wells on prepared plates, and incubated at 35^{0}C. Positive reaction was the formation of a bright pink halo extending at least 1 mm from the periphery of well. The results were noted after 4 hrs. of incubation and observed up to 18 hrs.

3.3.3.1.4. Phosphatase Test:

Requirements: Phenolphthalein diphosphate agar (Hi Media), liquor ammonia solution (SG 0.88).

Method: Test strain was streaked on the phenolphthalein diphosphate agar (PPA) plates and incubated for 18–20 hours at 37^{0}C. After incubation 01–02 ml ammonia solution was placed in the lid of petridish

and culture plate was inverted above it. Bright pink colonies were considered as positive. The test was performed using the method of Agarwal *et al.*, 2003.

3.3.3.1.5. β- Lactamase Production:

All the isolates of *Staphylococcus aureus* were tested for β-lactamase production using iodometric method.

Iodometric Method

Penicillinase hydrolyses penicillin to penicilloic acid, which reduce iodine and reverse the formation of the blue colour when latter complexes with starch. The presence of penicillinase in a test system is shown by decolorization of Starch-iodine complex The test was performed using the method of Sykes (1978).

Requirements:

Starch solution: 1% soluble starch solution was prepared by dissolving starch at 100^0C.

Iodine Reagent:

Iodine	2.03gm
Potassium Iodine	5.32gm
Distilled Water	100ml

Method:

A heavy suspension (about 10^9 colony units / ml) was prepared in wells of a microtitre plate from an overnight culture of test organism in

0.1 ml solution of benzylpencillin (6 mg/ ml in 0.1 mol/liter phosphate buffer, pH 7.3). The control tests with penicillinase negative and positive cultures were also set up. The microtitre plate was incubated for one hour at 37^{0}C, and then two drops of freshly prepared 1% solution of soluble starch was added to each well. In positive test, the blue colour was lost rapidly (within 10 seconds). In negative test blue colour persisted for at least 10 minutes.

3.3.3.2. Biochemical Tests:

All the isolates were subjected to various biochemical tests:

a. Production of acid from Sugars:

Each isolate was tested for production of acid from glucose and mannitol (both aerobically and anaerobically) and from trehalose (aerobically).

Method: Carbohydrate test solution were inoculated by test culture from nutrient agar plate and incubated overnight at 37^{0}C. Reddish pink colour of the medium indicates positive reaction.

b. Hugh and Leifson's O/F test:

Hugh and Leifson's O/F test was performed to see the ability of test strain to produce acid from glucose aerobically and / or anaerobically.

Method: Using a sterile straight wire test organism was stab inoculated throughout the length of sugar tube in duplicate. After inoculation the surface of one tube was covered with a 1-2 inch layer of sterile liquid paraffin. The tubes were then incubated at 37^{0}C for 5 days. Development of yellow colour indicated acid production. Acid production in the open

tube only indicated oxidative utilization of carbohydrate. Acid production both in open (with paraffin) and sealed tube indicated fermentation of sugar (1953).

c. Acetoin Production : Voges-Proskauer test:

Requirements:

Glucose Phosphate Peptone water

40% Potassium Hydroxide (KOH)

5% α-naphthol in absolute alcohol

Method: A large drop of broth culture of the strain under test was inoculated into 2 ml glucose phosphate peptone water tube and incubated at 30^0C for 18-48 hours.

The presence of acetoin production was detected by Barritt's method (1936):

1 ml of 40% Potassium hydroxide and 3 ml of 5% solution of α-naphtol in ethanol was added and shaken vigorously for at least 30 seconds. Positive reaction was indicated by the development of pink colour with in 5-10 minutes, becoming rose red colour in 30 minutes. The test was performed using the method of Mackie and McCartney, 2000.

d. Bacitracin Sensitivity:

Bacitracin sensitivity was done to differentiate between Micrococci and Staphylococci. *S. aureus* were resistant to bacitracin.

Resistance to bacitracin was tested on Mueller-Hinton agar medium (Hi Media) with 0.04 units of bacitracin disc (Hi Media). Resistance to bacitracin was reported when zone of inhibition of growth

was found less than 10 mm.

e. Novobiocin Sensitivity:

Resistance to Novobiocin distinguishes *S. saprophyticus* from other Staphylococci. Sensitivity to novobiocin was tested on Mueller-Hinton agar medium (Hi Media) with paper disc (7mm diameter) containing 5 µg of novobiocin. Sensitivity to novobiocin was reported when zone of inhibition was ≥15 mm in diameter.

3.3.3.3. Antimicrobial drug sensitivity test:

(a) Antibiotic sensitivity test was done by the disc diffusion method as proposed by Kirby Bauer (1961).

Requirements:

(i) Media:

Mueller-Hinton Agar (Hi Media)

Mueller-Hinton agar supplemented with an additional 5% NaCl (for Methicillin sensitivity)

(ii) Antibiotic discs (Hi-Media): Commercially available antibiotic discs obtained from (Hi Media) were used for antibiotic sensitivity testing. The antibiotics and their contents were:

Antibiotics	Abbreviation	Disc potency
Penicillin G	P	10 units
Amikacin	Ak	30 µg
Amoxycillin	Ac	30 µg

Chloramphenicol	C	30 µg
Ciprofloxacin	Cf	5 µg
Co–Trimoxazole	Co	25 µg
Cefoclor	Cj	30 µg
Ceftriaxone	Ci	30 µg
Cefepime	Cpm	30 µg
Ceftazidime	Ca	30 µg
Erythromycin	E	30 µg
Tetracycline	T	30 µg
Gentamycin	G	10 µg
Oxacillin	Ox	1 unit
Vancomycin	Va	30 µg
Teicoplanin	Te	30 µg

All antibiotic discs were stored in refrigerator. On removal from the refrigerator for use, the vials were left at room temperature for about an hour to allow the temperature to equilibrate, thus preventing the amount of condensation that occurs immediately after. Before use each lot of antibiotic disc was tested with standard strain of *S. aureus* 6571.

The results were interpreted according to the standard table provided by the supplier.

(iii) Standard control Strains:

Oxford *S. aureus* 6571

S. aureus (MRSA) in-house control

(iv) Test strains (Bacterial inoculum)

(v) Opacity Standard (0.5 McFarland)

(vi) Sterile Nontoxic cotton swabs.

Inoculum:

Test strains of *Staphylococcus aureus* and standard oxford *S. aureus* 6571 were isolated on nutrient agar. 4-5 identical colonies were picked up from both strains (standard and test strains) and inoculated in 5ml of nutrient broth separately and incubated at 37^0C for 4-6 hours. The density of the suspension was compared with the opacity standard tube i.e. 0.5 McFarland standard.

Method:

Drug sensitivity test for all antibiotics was carried out on Mueller-Hinton agar plate (Hi Media). While, for methicillin resistance oxacillin discs were used on Mueller-Hinton agar supplemented with 5% NaCl and incubated for 18-24 hour at 35°C.

The test strain was applied on the surface of the Mueller-Hinton agar plate using sterile swabs and allowed to dry for 10 minutes at room temperature. The antibiotic discs having the standard strength were lightly pressed on the surface of agar so as to ensure firm even contact of the disc with the seeded agar. Care was also taken to make sure that the placed discs straddle the gap uniformly at both sides and are about 1 cm

away from the rim of the plate. The plates were then incubated for overnight at 37^0C.

(b) Oxacillin agar screening (6µg/ml)

Inoculum: 2-5 colonies from overnight growth on a non-selective agar medium were suspended in broth or saline to a turbidity that matched with 0.5 Mc Farland standard. The suspension was diluted 1: 10 times and was adjusted to 10^7 cfu/ml.

Method: Plates were inoculated within 30 minutes after preparation of the inoculum since longer delay may lead to changes in inoculum size. By using a pipette, 0.001 to 0.002 ml of suspension of 10^7 cfu/ml was delivered to agar surface resulting in the final desired inoculum of approximately 10^4 to 10^5 cfu per spot. After inoculation plates were incubated for full 24 hrs. at 35^0C.

Interpretation: The presence of more than one colony or a haze of growth was considered as resistanct.

3.3.3.4. Bacteriophage typing:

Phage typing was done by the standard method described by Blair and Williams (1961) at National Staphylococcal Phage Center, Department of Microbiology Maulana Azad Medical College, New Delhi. For the propagation of the phages, testing of phage filtrate and typing the strains of *Staphylococcus aureus* isolates the propagating strain was first sub cultured onto a blood agar plate. A single colony was picked up and the phage pattern was checked using the 23 phages of the basic set at routine test dilution (1 RTD) and 100x RTD.

Propagation of the phages:

The phages were propagated on their homologous propagating strains. The aim of propagation was to produce a phage suspension of adequate dilution.

Propagation methods:

(a) Propagation in liquid medium:

The following phages were propagated in liquid medium: 55, 71, 53, 95 and 96.

(b) Propagation in soft agar:

The following phages namely 29, 52, 52A, 79, 80. 3A, 3C, 6, 42E, 47, 54, 75, 77, 83A, 84, 85, 81, and 91 were propagated in soft agar.

Determination of RTD: The phage preparation was diluted in ten-fold step. One drop of each dilution was then applied to a nutrient agar plate spread with the homologues propagating strain. The plate was incubated at 30°C overnight. The RTD (routine test dilution) was taken as the highest dilution of a phage that gave just less than confluent lysis.

Determination of 100 x RTD: The phage preparation was diluted in 100-fold steps. One drop of each dilution was then applied to a nutrient agar plate spread with the homologues propagating strain. The plate was incubated at 30°C overnight. The 100x RTD (routine test dilution) was taken as the highest dilution of a phage that gave just less than confluent lysis.

Performance of phage typing:

The basic set of phages, consisting of 23 phages was used for typing the strains of *Staphylococcus aureus*.

A Single colony of *Staphylococcus aureus* was inoculated into 5ml nutrient broth and incubated at 37° C for 4 to 6 hours. This culture was used to inoculate 4-inch peteridishes containing nutrient agar supplemented with 0.04% fused calcium chloride. The plates used for typing were freshly made and dried for 45 minutes. The broth culture of the test strain was added on the surface of the plate with a Pasteur pipette and the excess broth was pipetted off. These were then left for drying with lids open for one hour at room temperature.

The phages were loaded in a block containing 27 wells, using sterile Pasteur pipettes. Phages were delivered from the loading chamber to agar plate with the help of mechanical multi-loop/ phage applicator.

After each application, the prongs of the applicator were dipped in spirit and were sterilized by flaming. After the charged phages had dried, the plates were incubated at 30°C for 18 hrs. The readings were taken on the following day.

Interpretation of the lytic pattern:

The plates were examined by indirectly transmitted light against a dark background and read semi-quantitatively.

Strains that were non-typeable at 1 RTD were typed at 100xRTD. Non-typeablity was recorded when the strains was non-typeable at 100x RTD.

The phage patterns were recorded as follows:

++, CL = Confluent lysis (Strong reaction)

\+ =Moderate lysis

± = Weak lysis

o = inhibition reaction

NT = Not typeable

Conventional Phages

Lytic Group of Phages	Designation of Phages
I	29,52,52A,79,80
II	3A, 3C, 55, 71
III	6, 42E, 47, 53, 54, 75, 77, 83A, 84, 85
Non – allocated	81, 94, 95, 96

TYPING OF METHICILLIN-RESISTANT *Staphylococcus aureus* BY MRSA PHAGES

All methicillin resistant staphylococcal stains were phage typed using 9 supplementary MRSA phages.

The 9 phages used were:

M3, M5, M12, M8, MR25, 622, C30, C33, C38

3.4. Tests for genotypic characterization:

Genotypic characterization was done using polymerase chain reaction for thermostable nuclease gene, coagulase gene, enterotoxin genes and methicillin gene.

3.4.1. Polymerase Chain Reaction (PCR):

Standardization of PCR:

Standardization of Polymerase Chain Reaction (PCR) was done using the standard strains of *S. aureus* obtained from IVRI, Izatnagar.

3.4.1.1. Template DNA Preparation / Extraction:

Various methods of cell lysis and release / extraction of DNA as described below were compared for their use in PCR assay.

3.4.1.1.1. Genomic DNA Extraction:

By Phenol Chloroform Extraction:

All isolates under study were cultured in Tryptone Soya Broth (TSB, oxide Ltd. England) at 37°C for 18h. Bacteria were pelleted @ 8000 rpm for 10 mins. Pellet was resuspended in 2ml TE buffer. 40 µl of lysozyme (50 mg / ml) was added, vortexed to mix properly and incubated at 37°C for 1h. Then 400 µl SDS (10%) and 30 ml Proteinase-K (20mg / ml) was added and incubated at 37°C for 2h. After incubation, 800 µl of NaCl (5M) and 800 µl CTAB (10%, Preheated at 65°C), were added and incubated in water bath at 65°C for 30 minutes. The aqueous phase was extracted with equal amount of Phenol + Chloroform (1:1). The phenol-chloroform extraction step was repeated as to the extracted aqueous phase, 0.6 volume of isopropanol and 0.1 volume of 3M sodium

acetate (pH 5.2) were added. The components were mixed properly, and kept at -20°C for 8-10h. Centrifugation at 20,000 rpm for 10 minute was performed to collect DNA pellet. The pellet was washed twice with 70% ethanol and air-dried. Finally the pellet was dissolved the pellet in 400 µl of TE buffer containing 20 µg RNase / ml (MBI Fermentas) and kept at 65 °C for 1 hr. Subsequently cooled and stored at -20°C till further use.

3.4.1.1.2. By Using Bacterial Cell Lysate:

(a) By Triton X – 100:

As per the method described by Wang *et. al*, (1992), cells were pelleted and suspended in 50 -100 µl of 1% Triton X-100. It was then heated in a boiling water bath for 5 minutes and snaps chilled.

(b) By SDS:

The bacterial cells were pelleted and suspended in about 20 µl of 0.05% SDS and heated in a boiling water bath for 5 minutes and snap chilled.

(c) By Sonication:

About 100-200 µl of BHI broth culture was subjected to sonication and was used in PCR.

(d) By Boiling and Chilling:

In this method about 0.5-1.0 ml of BHI broth culture was subjected to vigorous heating in a boiling water bath for 10 minutes and then snap chilled. From this about 5 µl was used as a template in PCR.

For standardization with DNA extraction, initially all the methods described above were applied on the standard strains. As all these

methods showed comparable results, we subsequently followed the boiling and chilling method for the DNA extraction from the test strains.

3.4.2. Specificity of the PCR:

About 0.5µl of overnight BHI cultures was subjected to heating in a boiling water bath and then snap chilled in ice. The PCR was performed as described below and agarose gel electrophoresis as described in section 3.5, and observed for any product generated from other bacteria.

3.4.3. PCR amplification of thermostable nuclease gene (Nuc):

PCR amplification was performed by using the primers as used by Brakstad *et al.,* (1992) with slight modifications. The thermostable nuclease gene (nuc) was amplified by standard PCR protocol. The template was prepared as described in 3.4.1.1.2 (d). Sequences of the primers used were

nuc 1- G C G A T T G A T G G T G A T A C G G T T

nuc 2 - A G C C A A G C C T T G A C C A A C T A A A G C

Size of the amplified product – 447 bp

Reactions were carried out on 50µl as follows.

DNA	-	5µl
10x PCR buffer	-	5 µl
$MgCl_2$ (1.5mM)	-	1.5 µl
dNTP's (2mM)	-	5 µl
Primers (10 pmol/ µl)	-	N1– 2.0 µl
		N2– 2.0 µl
Taq DNA polymerase (1u/µl Labware, USA)	-	0.4 µl
Milli Q water to make volume 50 µl.		

The PCR tube containing the reaction mixture was flash spun in a micro centrifuge to get the reactants at the bottom. The reactions were performed in a master cycler Gradient thermocycler (Eppendrof, Germany) with a preheated lid.

The PCR programme included denaturation at 94°C for 5 minutes followed by 34 cycles of denaturation (94°C for 1 minute), annealing (55°C for 1 minute) and extension (72°C for 1 minute). Final extension was carried out at 72°C for 10 minutes After the reaction, tubes with PCR products were held at 4°C until further analysis / confirmation by agarose gel electrophoresis (1.5% agarose) described as in 3.5. gene Ruler™ 100 bp DNA ladder was used as molecular weight marker.

3.4.4. PCR and nested PCR amplification of coagulase gene (*coa*):

PCR and nested PCR were performed for amplification of coagulase gene.

3.4.4.1. PCR amplification of coagulase gene (*coa*):

By using the primers Goh *et al.*, (1992) with slight modification the coagulase gene (*coa*) was amplified by standard PCR protocol. Template was prepared as described in 3.4.1.1.2 (d). Sequences of the primers used were

Coa 1- ATACTCAACCGACGGACACCG

Coa 2- GATTTTGGATGAAGCGGATT

Reactions were carried out on 50 µl as follows

DNA	-	4µl
10x PCR buffer	-	5µl
$MgCl_2$ (1.5mM)	-	1.5 µl
dNTP's (2mM)	-	5µl
Primers (10 pmol /µl)	-	coa 1 – 2.00 µl
		coa 2 – 2.00 µl
Taq DNA polymerase	-	0.4 µl

(1unit / reaction, Lab ware, USA)

Milli Q water to make volume 50 µl.

The PCR tube containing the reaction mixture was flash spun in a micro centrifuge to get the reactants at the bottom. The reactions were performed in a master cycler Gradient thermocycler (Eppendrof, Germany) with a preheated lid.

The PCR programme included denaturation at 94°C for 5 minutes followed by 34 cycles of denaturation (94 °C for 1 minute), annealing (56 °C for 1 minute) and extension (72°C for 1 minute). Final extension was carried out at 72 °C for 10 minutes. After the reaction, tubes with PCR products were held at 4°C until further analysis / confirmation by agarosegel electrophoresis (2% agarose) described as in section 3.5. Gene Ruler™ 100 bp DNA ladder plus was used as molecular weight marker.

3.4.4.2. Nested PCR amplification of coagulase gene (*coa*):

Nested PCR was standardized as per method described by Goh *et al.* (1992) with slight modifications. All isolates were tested in duplicates. Depending on the intensity of the primary amplicon generated by primer set no. 1, 2 microlitre of undiluted to 1:10 diluted primary

amplicon was taken for nested PCR. The internal primer set no. 2 was used to generate nested PCR products. Sequences of the primers used were

coa N – 1 CGAGACCAAGA[illegible]TCAACAAG

coa N – 2 AAAGAAAACCA[illegible]TCACATCA

Reactions were carried out on 50 µl as follows.

Size of the amplified products - Ranged between 440 bp to 915 bp

Ist PCR product	-	2.0µl
10x PCR buffer	-	5µl
$MgCl_2$ (1.5mM)	-	1.5 µl
dNTP's (2mM)	-	5µl
Primers (10 pmol / µl)	-	coa 1- 2.0µl
		coa 2- 2.0µl
Taq DNA polymerase	-	0.4 µl

(1u/ µl, Lab ware, USA)

Milli Q water to make volume 50 µl.

The PCR tube containing the reaction mixture was flash spun in a micro centrifuge to get the reactants at the bottom. In this study the previous PCR product used as template. The reactions were performed in a master cycler Gradient thermocycler (Eppendrof, Germany) with a preheated lid.

The PCR program and other procedures were same as described earlier for the first amplification of *coa* gene.

3.4.4.3. PCR restriction analysis:

Restriction enzyme digestion:

For the confirmation of the PCR product generated by primer set no. 1, restriction digestion with *Alu 1* was done. To about 10µl of nested PCR product, 6 unit (0.6 µl) of *Alu 1* was added (Bangalore, Genie) with 2µl of 10x buffer and the volume was made up to 20 µl with autoclaved milli Q water and incubated at 37°c for overnight. The enzyme activity was stopped by adding 4µl of 6x loading dye. Then the digested product was subjected to agarose gel electrophoresis (2% agarose) and observed for the products of desired molecular weight. All isolates were again tested in duplicate.

3.4.5. PCR amplification of enterotoxin gene (SEA, SEB and SEC):

A total of 202 isolates (102 human clinical isolates and 100 animal-origin isolates) were tested for production of enterotoxin A, enterotoxin B and enterotoxin C. PCR amplification for enterotoxin genes were performed by using the methods described by Johnson *et al.* (1991) (for SEA and SEB) while the method of Chen *et al* (2000) was used for SEC with slight modification. Templates were prepared as described in 3.4.1.1.2 (d). Sequences of the primers used were

For SEA SEA – 1 TTGGAAACGCTTAAAACGAA

SEA – 2 GAACCTTCCC ATCAAAAACA

Size of the amplified product – 120 bp

For SEB SEB – 1 TCGCATCA AACTGACAAACG

SEB – 2 GCAGGTAC TCTA AAGTGCC

Size of the amplified product – 478 bp

For SEC SEC – 1 ACATTAGTGATAAAAAACTGAAA

SEC – 2 TTGTAAGTTCCCATTATCAAAGTG

Size of the amplified product – 234 bp

Reactions were carried out on 50µl as follows

DNA	-	4µl
10x PCR buffer	-	5µ
$MgCl_2$ (1.5mM)	-	1.5µl
d NTP'S (2mM)	-	5 µl
Primers (10 pmol / µl)	-	forward – 2.0 µl
		reversed – 2.0 µl
Taq DNA polymerase	-	0.4 µl
(1u / µl, Labware, USA)		

Milli Q water to make volume 50 µl.

The PCR tube containing the reaction mixture was flash spun on a micro centrifuge to get the reactants at the bottom. The reactions were performed in a master cycler Gradient thermocycler (Eppendrof, Germany) with a preheated lid.

The PCR programme included denaturation at 94°C for 5 minutes followed by 34 cycles of denaturation (94 °C for 30 seconds), annealing (55 °C for 30 seconds) and extension (72°C for 20 seconds). Final extension was carried out at 72°C for 10 minutes. The PCR products were characterized by gel electrophoresis on 2.5% agarose gel (Q. Biogene, USA) as described in 3.5.

Gene Ruler™ 100 bp DNA ladder was used as molecular weight marker.

3.4.6. PCR amplification of methicillin resistance gene (*mec A*):

By using the primers as used by Prasad *et al.* (2000), with slight modification, the methicillin resistant gene (*mec A*) was amplified by the standard PCR protocol. Template was prepared as described in 3.4.1.1.2 (d). Sequences of the primers used were

mec A 1 – A G T T G T A G T T C T C G G G T T T G

mec A 2 – A G T G G A A C G A A G G T A T C A T C

Size of the amplified product – 604 bp

Reactions were carried out on 50 µl as follows.

DNA	-	10µl
10x PCR buffer	-	5µl
$MgCl_2$ (1.5mM)	-	1.5 µl
dNTP's (2mM)	-	5µl
Primers (20 pmol / µl)	-	mec A1 - 2.0µl
		mec A2 - 2.0µl
Taq DNA polymerase (1unit/reaction, Lab ware, USA)	-	0.4µl

Milli Q water to make volume 50 µl.

The PCR tube containing the reaction mixture was flash spun in a micro centrifuge to get the reactants at the bottom. The reactions were performed in a master cycler Gradient thermocycler (Eppendrof, Germany) with a preheated lid.

The PCR programme includes denaturation at 94°C for 5 minutes followed by 34 cycles of denaturation (94°C for 1 minute), annealing

(54°C for 1.5 minutes) and extension (72°C for 1 minute). Final extension was carried out at 72°C for 10 minutes. After the reaction, tubes with PCR products were held at 4°C until further analysis / confirmation by agarose gel electrophoresis (2% agarose) described as in 3.5. Gene Ruler™ 100 bp DNA ladder was used as molecular weight marker.

3.5. Agarose gel electrophoresis:

Agarose gel was prepared by boiling molecular biology grade agarose (Bangalore, Genie) in 0.5-x TBE buffer to dissolve it completely. After cooling it to about 50°C, ethidium bromide (SRL) was added to the agarose solution to a final concentration of 0.5 µg / ml. Before dissolving the agarose, the gel-casting platform was placed on a leveled surface and the open sites were sealed with adhesive tape. The gel comb was then placed across the gel-casting platform, so that the teeth of the comb remained 1mm above the base of the platform. The molten agarose was then poured on to the gel-casting platform and it was kept undisturbed for about an hour to solidify the gel. After the gel solidified the comb was taken out and adhesive tape was removed The set gel with the gel-casting platform was then submerged in the elect ophoresis tank with the wells at the cathode end of the tank with sufficient quantity (about 1 mm level) of electrophoresis buffer (TBE, 0.5x) above the surface of the gel.

About 10 µl of PCR products was mixed with 2 µl of bromophenol blue (6x) loading dye and loaded into the well. Electrophoresis was performed at 6 V/cm and the progress of mobility was monitored by the migration of the dye. At the end of electrophoresis the gel was visualized under UV transilluminator / gel documentation system for the bands of desired molecular weight.

RESULTS

4. RESULTS

In the present study out of 3514 **human clinical specimens** 1417-yielded bacterial growth and of which 409 were *S. aureus*. Of these 409 *S. aureus* isolates, 102 were randomly selected for further study. Similarly, 1219 samples of **animal-origin**, including 213 **milk samples as animal clinical specimens** and 1006 **food samples** were cultured and 918-yielded bacterial growth. Of which 401 were *S. aureus* of which 100 animal origin food isolates were randomly selected for further study. Out of 102 *Staphylococcus aureus* studied from **human clinical specimens**, 38 were from pus samples, 10 from urine samples, 1 from CSF, 2 from I/V catheter tips, 3 from body fluid, 3 from ear swab, 3 from throat swab, 14 from conjuctival swab, 14 from cervical swab, 7 from stool samples, 6 from vomitus samples and 1 from semen specimen. Of the 100 isolates of *S. aureus* from **animal origin food,** 29 were obtained from raw milk (clinical isolates of animal origin), 4 from chamcham (a sweet prepared from milk), 3 from other sweets, 9 from khoa (milk concentrate used in sweets), 6 from paneer (cottage cheese), 17 from raw goat meat, 20 from raw buffalo meat, 1 from kabab (Meat cutlet) and 1 from salami sample.

4.1. Characterization of *Staphylococcus aureus:*

Phenotypic characterization:

All the isolates were phenotypically characterized using conventional methods, in direct microscopy examination 89 human clinical samples showed Gram-positive cocci in clusters.

All isolates from human clinical specimens and animal-origin samples were found positive for catalase test, DNase test, phosphatase test, tube coagulase test and were oxidase negative. Out of 102 human clinical and

100 animal-origin samples, 100 (98.03%) and 98 (98%) were found positive for slide coagulase test respectively; all (100%) strains showed fermentation of glucose, mannitol and trehalose. Acetoin production was seen in all the human and animal-origin strains. All the 202 (100%) strains of clinical and animal-origin isolates were found sensitive to novobiocin and resistant to bacitracin.

4.2. Further characterization of *Staphylococcus aureus* strains:

The characterization was mainly based on:

- Antibiotic susceptibility
- Bacteriophage typing

4.2.1. Antibiotic susceptibility testing:

To detect the susceptibility of *Staphylococcus aureus* isolates against various antibiotics, *in vitro* antibiotic sensitivity was carried out by disc diffusion test according to the method of Bauer *et al* (1961). In this study 16 commonly used antibiotics, namely, amoxycillin, penicillin, cotrimoxazole, chloramphenicol, tetracycline, erythromycin, oxacillin, ciprofloxacin, cefaclor, ceftriaxone, ceftazidime, cefepime, gentamicin, amikacin, vancomycin and teicoplanin were used (fig- 1).

(i) Antibiotic susceptibility of human clinical isolates:

Antibiotics sensitivity of 102 **human clinical isolates** revealed that maximum number of isolates were resistant to penicillin 100 (98.03%) followed by cotrimoxazole 71 (69.61%), tetracycline 70 (68.63%), amoxycillin 66 (64.7%), ciprofloxacin 62 (60.79%), erythromycin 56 (54.9%), amikacin 35 (35.3%), oxacillin 33 (32.35%), cefaclor 33 (32.35%), ceftriaxone 33 (32.35%), ceftazidime 33 (32.35%), cefepime

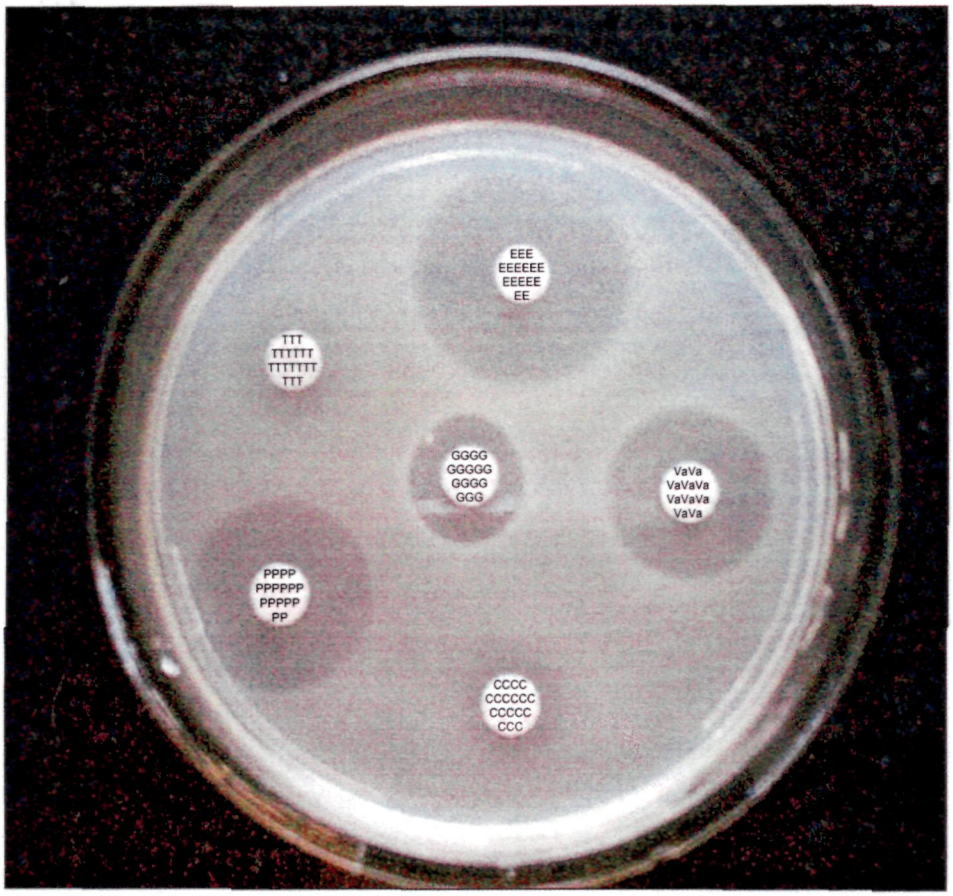


Fig - 1 Photograph showing antimicrobial sensitivity on Meuller-Hinton agar medium

33 (32.35%), chloramphenicol 24 (23.53%) and gentamicin 28 (27.45%) in descending order (fig- 2) while none of the isolate was found resistant to vancomycin and teicoplanin (Table - 1).

In this study, methicillin resistance was noticed in 33 (32.35%) isolates by disc diffusion method and in 32 (31.35%) by agar screen method using 6 µg oxacillin while 77 (67.65%) of clinical isolates were found methicillin sensitive *Staphylococcus aureus.*

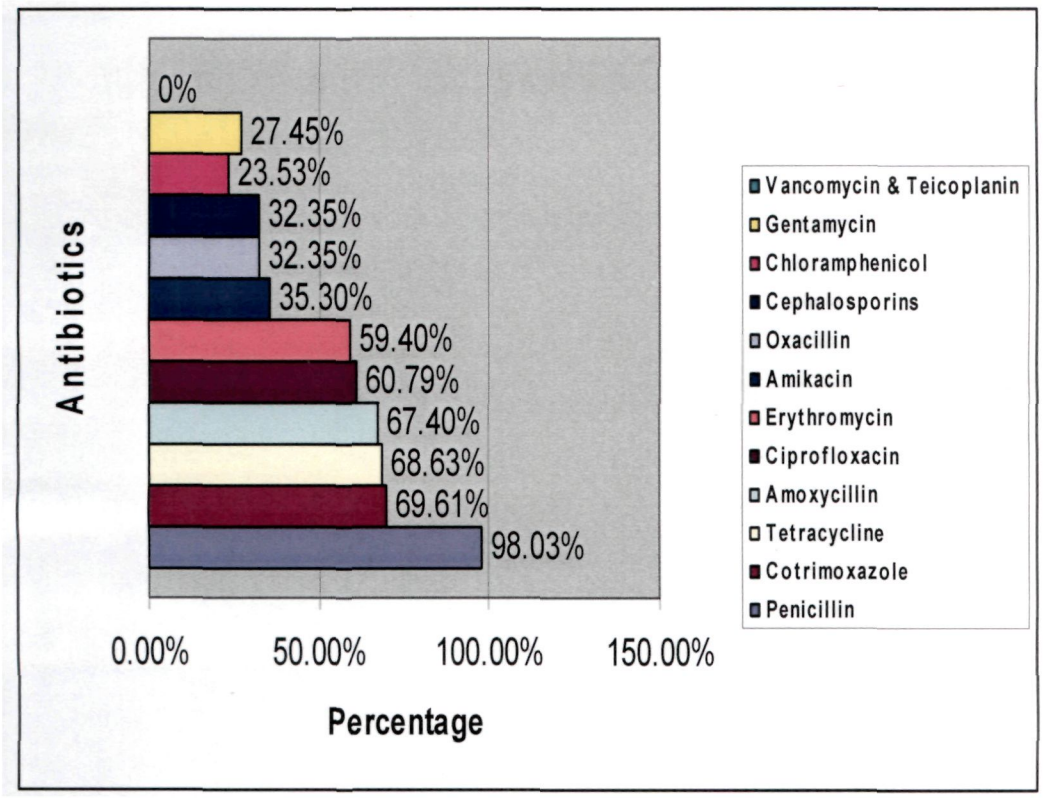


Fig- 2. Bar diagram showing antibiotic resistance profile of *Staphylococcus aureus* isolated from different human clinical specimens

Table-1 Antibiotic sensitivity profile of *Staphylococcus aureus* isolated from different clinical specimens

Sensitive to	*S. aureus Isolated from*												Over all %
	Pus n = 38	Urine n = 10	Cx.Swab n = 14	Semen n = 1	Throat Swab n = 3	Ear Swab n = 3	Conj. Swab n = 14	Stool n = 7	Vomitus n = 6	CSF n = 1	Body Fluids n = 3	Catheter Tips n = 2	
Amikacin	16(42.11)	10(100)	10(71.43)	1(100)	2(66.67)	2(66.67)	10(71.43)	6(85.71)	6(100)	0(0)	2(66.67)	1(50.0)	64.7
Amoxicillin	1(2.63)	10(100)	14 (100)	0(0)	0(0)	0(0)	3(21.43)	3(42.86)	4(66.66)	0(0)	1(33.33)	0(0)	35.3
Cefaclor	24(63.16)	6(60.0)	10(71.43)	1(100)	2(66.67)	2(66.67)	10(71.43)	6(85.71)	5(83.33)	0(0)	2(66.67)	1(50.0)	67.65
Cefepime	24(63.16)	6(60.0)	10(71.43)	1(100)	2(66.67)	2(66.67)	10(71.43)	6(85.71)	5(83.33)	0(0)	2(66.67)	1(50.0)	67.65
Ceftriaxone	24(63.16)	6(60.0)	10(71.43)	1(100)	2(66.67)	2(66.67)	10(71.43)	6(85.71)	5(83.33)	0(0)	2(66.67)	1(50.0)	67.65
Ceftazidime	24(63.16)	6(60.0)	10(71.43)	1(100)	2(66.67)	2(66.67)	10(71.43)	6(85.71)	5(83.33)	0(0)	2(66.67)	1(50.0)	67.65
Chloramphenicol	34(89.47)	5(50.0)	8(57.14)	1(100)	0(0)	3(100)	12(85.71)	5(71.43)	5(83.33)	1(100)	3(100)	1(50.0)	76.47
Ciprofloxacin	18(47.37)	1 (10)	8(57.14)	1(100)	0(0)	0(0)	4(28.50)	5(71.43)	2(33.33)	0(0)	1(33.33)	0(0)	39.21
Cotrimoxazole	11(28.95)	1(10.0)	6(42.86)	0(0)	1(33.33)	0(0)	3(21.43)	4(57.14)	1(16.67)	1(100)	2(66.67)	1(50)	30.39
Erythromycin	23(60.53)	1(10.0)	10(71.43)	1(100)	2(66.67)	2(66.67)	2(14.29)	1(14.29)	1(16.66)	1(100)	1(33.33)	1(50.0)	45.1
Gentamycin	24(63.16)	1(10.0)	13(92.86)	1(100)	3(100)	3(100)	14(100)	6(85.71)	5(83.33)	1(100)	2(66.67)	1(50.0)	72.55
Oxacillin	24(63.16)	6(60.0)	10(71.43)	1(100)	2(66.67)	2(66.67)	10(71.43)	6(85.71)	5(83.33)	0(0)	2(66.67)	1(50.0)	67.65
Penicillin	1(2.63)	0(0)	0(0)	1(100)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1.96
Tetracycline	20(52.63)	1 (10.0)	3(21.43)	1(100)	1(33.33)	0 (0)	3(21.43)	0(0)	1(16.67)	0 (0)	1(33.33)	1(50.0)	31.37
Vancomycin	38(100)	10(100)	14(100)	1(100)	3(100)	3(100)	14(100)	7(100)	6(100)	1(100)	3(100)	2(100)	100
Teicoplanin	38(100)	10(100)	14(100)	1(100)	3(100)	3(100)	14(100)	7(100)	6(100)	1(100)	3(100)	2(100)	100

Figures in parentheses indicate percentage

(ii) Antibiotic susceptibility of animal-origin isolates:

The isolates from **animal-origin samples** showed resistance to penicillin 93 (93%), erythromycin 51 (51%), tetracycline 49 (49%), ciprofloxacin 39 (39%), cotrimoxazole 54 (54%), chloramphenicol 34 (34%), amikacin 27 (27%), oxacillin (27%), cefaclor 27 (27%), ceftriaxone 27 (27%), ceftazidime 27 (27%), cefepime 27 (27%), gentamicin 20 (20%) and amoxycillin 19 (19%) in descending order (fig- 3). One isolate was found susceptible to all the 16 antibiotics and none of the strain was found resistant to vancomycin and teicoplanin (Table - 2).

In this study, methicillin resistance was noticed in 27 (27%) isolates by disc diffusion method and in 26 (26%) by agar screen method using 6 µg oxacillin while 73 (73%) of animal-origin isolates were found methicillin sensitive *Staphylococcus aureus.*

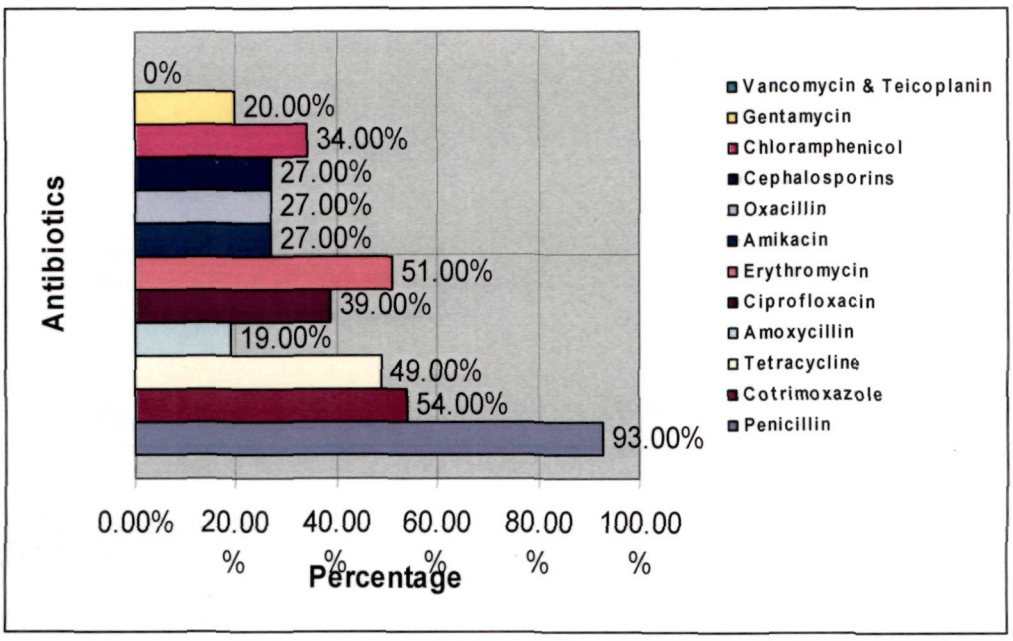


Fig- 3. Bar diagram showing antibiotic resistance profile of *Staphylococcus aureus* isolated from different animal-origin samples

Table- 2. Antibiotic sensitivity profile of *Staphylococcus aureus* isolated from different animal-origin food samples

Sensitive to	*S. aureus* isolated from					Overall Percentage
	Milk n = 29	Milk Product n = 22	Buffalo Meat n = 20	Goat Meat n = 27	Meat Products n = 2	
Amikacin	18 (62.1)	17 (77.3)	18 (90.0)	18 (66.7)	2 (100)	73
Amoxacillin	21 (72.4)	19 (86.4)	20 (100)	19 (70.4)	2 (100)	81
Cefaclor	14 (48.28)	18 (81.81)	18 (90.0)	22 (81.48)	1 (50.0)	73
Cefepime	14 (48.28)	18 (81.81)	18 (90.0)	22 (81.48)	1 (50.0)	73
Ceftriaxone	14 (48.28)	18 (81.81)	18 (90.0)	22 (81.48)	1 (50.0)	73
Ceftazidime	14 (48.28)	18 (81.81)	18 (90.0)	22 (81.48)	1 (50.0)	73
Chloramphenicol	13 (44.9)	16 (72.7)	15 (75.0)	20 (74.1)	2 (100)	66
Ciprofloxacin	12 (41.4)	16 (72.7)	12 (60.0)	19 (70.4)	2 (100)	61
Co-trimoxazole	6 (20.7)	11 (50.0)	18 (90.0)	10 (37.0)	1 (50.0)	46
Erythromycin	19 (65.5)	3 (13.6)	10 (50.0)	15 (55.6)	2 (100)	49
Gentamycin	22 (75.9)	16 (72.7)	18 (90.0)	22 (81.5)	2 (100)	80
Oxacillin	14 (48.28)	18 (81.81)	18 (90.0)	22 (81.48)	1 (50.0)	73
Penicillin	3 (10.3)	1 (4.5)	0 (0)	1 (3.7)	2 (100)	7
Tetracycline	18 (62.1)	10 (45.5)	6 (30.0)	16 (59.3)	1 (50.0)	51
Vancomycin	29 (100)	22 (100)	20 (100)	27 (100)	2 (100)	100
Teicoplanin	29(100)	22(100)	20(100)	27(100)	2(100)	100

Figures in parentheses indicate percentage

(iii) Drug resistance patterns of human clinical isolates:

The drug resistance patterns of **human clinical isolates** showed resistance to two drugs in 3 (2.94%) isolates, three drugs in 10 (9.8%) isolates, four drugs in 18 (17.65%) isolates, five or six drugs in 25 (24.51%) isolates, seven or eight drugs in 16 (15.67 %) isolates whereas resistance to more than ten or more drugs was found in 30 (29.47%) isolates. None of the isolate was found resistant to a single drug (Table - 3).

Table- 3. Drug resistance patterns of *S. aureus* isolated from human clinical specimens

Resistant to	Patterns (n)	Total numbe(%)
Ten or more drugs	P Ac Ak E G Cf C Co Ox Cj Ci Ca Cpm(2) P Ac Ak E T Cf C Co Ox Cj Ci Ca Cpm(2) P Ac Ak E T G Cf Co Ox Cj Ci Ca Cpm(5) P AcAkETGCfCOxCjCiCaCpm(1) P AcAkETCfCOxCjCiCaCpm(1) P AcAkETCfCoOxCjCiCaCpm(2) P AcAkTGCfCoOxCjCiCaCpm(2) P AcAkETCCoOxCjCiCaCpm(2) P AcAkETGCfOxCjCiCaCpm(1) P AkETCfCoOxCjCiCaCpm(5) P AcAkETCfOxCjCiCaCpm(1) P AcAkGCfCoOxCjCiCaCpm(2) P AcAkCfCCoOxCjCiCaCpm(1) P AcAkGCfCoOxCjCiCaCpm(1) P AkECfCoOxCjCiCaCpm(1) E TCfCoOxCjCiCaCp n(1)	30(29.47%)
Seven or Eight drugs	PETCfCCoCpm(1) PAcETGCfCo(1) AcTCfCCoCjCpmOx(1) PAcAkECfCCo(1) PAcAkCfCCoOx(1) PAcAkETCfCo(8) PAcETGCCo(1) PETGCfCCo(1) PAcAkEGCfCo(1)	16 (15.67%)

Five or Six drugs	PAcGCfCo(1) PAcETCf(2) PAcTGCo(1) PAcETCo(2) PAcTCfCo(1) PAcAkTCo(2) PTCfCCo(1) PTAcCfCo(1) PTCfECo(1) PTCECo(1) PAcTCoCf(1) PAcECoCf(1) PETCoCf(2) PEAkTCfCo(2) PAkAcECf(1) PAkAcTC(1) PAkAcCCo(1) PAkECfCo(2) PAkAcETCo(1)	25 (24.51%)
Four drugs	PCAcCo(1) PCfCCo(1) PAkTCo(1) PAcCfCo(1) PAcET(3) PGET(3) PAkAcC(1) PTGCf(2) PAcET(2) PTGCo(1) PEAcCf(2)	18 (17.65%)
Three drugs	PAcCo(1) PTE(1) PCCo(6) PCAc(1) PCCf(1)	10 (9.80%)
Two drugs	P(3)	3 (2.94%)
Single drug	Nil	0 (0%)
Total		102 (100%)

Abbreviation Key:
P = Penicillin G, Ak = Amikacin, Ac = Amoxicillin, C = Chloramphenicol, Cf = Ciprofloxacin, Co = Co –Trimoxazole, Cj = Cefaclor, Ci = Ceftriaxone, Cpm = Cefepime, Ca = Ceftazidime, E = Erythromycin, T = Tetracycline, G = Gentamicin, Ox = Oxacillin

(iv) Drug resistance patterns of animal-origin isolates:

In **animal-origin samples** the drug resistance patterns showed resistance to one drug in 8 (8%), two drugs in 9 (9.0%) isolates, three drugs in 18 (18.0%) isolates, four drugs in 16 (16.0%) isolates, five or more than five drugs in 25 (25.0%) isolates whereas resistance to ten or more drugs was found in 23 (23.0%) isolates. One isolate was found sensitive to all drugs (Table - 4).

Table- 4. Drug resistance patterns of *S. aureus* isolated from animal-origin samples

Resistant to	Patterns (n)	Total numbers (%)
Ten or more drugs	PETGCfCCoAkOxCjCiCaCpm(2) PETCCfCoAcAkOxCjCiCaCpm(1) PETGCAcAkOxCjCiCaCpm(2) PETCCoAcAkOxCjCiCaCpm (2) PETGCoAcAkOxCjCiCaCpm (3) PETGCf Co Ox Cj Ci Ca Cp m(2) PETGCoOxCjCiCaCpm(1) PETCfCCoOxCjCiCaCpm(1) PTGCfCoOx Cj Ci Ca Cpm (1) PAkTECoOxCjCiCaCpm (1) ECfCoAcAkOxCjCiCaCpm(1) PETAc Ak Ox Cj Ci Ca Cpm(2) ETCfCAcAkOxCjCiCaCpm(1) ECCoAcAkOxCjCiCjCpm(2) TECfCoOxCCjCiCaCpm(1)	23 (23%)
Five or more drugs	PETG Cf Co Ac Ak (3) AkTGCoOxCjCiCaCpm(2) PAkETOxCjCiCaCpm(2) PTAk Ox Cj Ci Ca Cpm (1) PECfGCoC(2) ETCfCoAcAk(1) PETCAcAk(1) PTGCfCiCo(1) PETCfCo(2) PECfCCo(2) ETCAcAk (1) PETCfC (1) PAkECfC (2) PECAkCf (1) PETGCfC (3)	25 (25%)

Four drugs	AkTCfCo(2) PETC(1) PETCo(1) ETGCo(1) PAcET(2) GCfCCo(1) PGCCo(1) PTCCo(2) PEGCo(1) PECo(2) PGTC(2)	16 (16%)
Three drugs	ETC(1) ETCo(1) PTCo(2) PCfCo(1) PET(1) PGT(3) PTCf(1) PECf(4) TCCo(2) PEC(2)	18 (18%)
Two drugs	PAc(1) GT(3) ET(1) ECo(1) PT(1) PE(1) AkCf(1)	9 (9%)
Single drug	P(2) Cf(2) T(3) C(1)	8 (8%)
Total		99* (99%)

* 1 strain was found sensitive to all antibiotics.

Abbreviation Key:

P = Penicillin G, Ak = Amikacin, Ac = Amoxycillin, C = Chloramphenicol, Cf = Ciprofloxacin, Co = Co–Trimoxazole, Cj = Cefaclor, Ci = Ceftriaxone, Cpm = Cefepime, Ca = Ceftazidime, E = Erythromycin, T = Tetracycline, G = Gentamicin, Ox = Oxacillin

4.2.2. Bacteriophage typing of *Staphylococcus aureus* isolates from human clinical specimens and animal-origin samples:

(i) Routine bacteriophage typing of human clinical isolates: Bacteriophage typing was performed in 102 clinical isolates and 100 food isolates. Out of the 102 **human clinical isolates** tested, only 55 (53.9%) of isolates could be typed by the conventional set of phages at RTD (Table - 5).

Table-5. Bacteriophage typing of *Staphylococcus aureus* isolates from human clinical specimens at 1 RTD

Source	Total no. of isolates tested	1 RTD					
		Typeable groups				Mixed group	Typeable isolates
		I	II	III	Non allocated		
Pus	38	0	7	5		I & III = 3	15
Urine	10		1	3			4
Cx. Swab	14	2	2	5		I & II = 2	11
Semen	1						0
Conj Swab	14		4	3			7
Ear Swab	3		2				2
ThroatSwab	3		1	1			2
Stool	7			4		III&IV = 1 I & IV = 1	6
Vomitus	6		2	3			5
Body Fluid	3	1		1			2
Cathetertips	2		1				1
CSF	1						0
Total	102	3 (2.94%)	20 (19.6%)	25 (24.5%)		7 (6.9%)	55 (53.9%)

Distribution of isolates into phage groups revealed that maximum number of isolates 25 (24.51%) were typed in group III followed by 20 (19.61%) in group II, 7 (6.9%) were in mixed group and 3 isolates (2.94%) in group I. No isolate was found in the nonallocated group. 47 (46.07%)

isolates remained untypeable at 1 RTD. On testing these 47 un-typeable isolates at 100 RTD, they showed 16.6 7% (17/102) further typeablity. Among these 17 isolates typed at 100 R' 'D, 10 isolates were methicillin resistant. Majority of these isolates b.longed to group III (23.4%) followed by 4 (8.5%) in mixed group and 2 (4.3%) in group II (Table-6).

Table- 6. Bacteriophage typing of *Staphylococcus aureus* isolates from human clinical specimens at 100 RTD

Source	Total no. of isolate tested	100 RTD					
		Typeable groups				Mixed group	Typeable group
		I	II	III	Non allocated		
Pus	23		2	3		I & III = 3	8
Urine	6			2			2
Cx - Swab	3			3			3
Semen	1			1			1
Conj Swab	7						0
Ear Swab	1						0
Throat Swab	1						0
Stool	1			1			1
Vomitus	1			1			1
Body Fluid	1					I & III = 1	1
Drain Tip	1						0
CSF	1						0
Total	47		2(4.3%)	11(23.4%)		4(8.5%)	17(36.2%)

The total typeability of clinical isolates at 1 RTD and 100 RTD was found to be 70.58% (72/102). Typeability pattern of isolates obtained from different human clinical samples is shown in table- 7.

Table- 7. Bacteriophage typing of human clinical isolates of *Staphylococcus aureus* from various samples at (1 RTD and 100 RTD) combined

Source	Total no.of isolates tested	Typeable no. of isolates (%)	Nontypeable no. of isolates (%)
Pus	38	23 (60.5%)	15 (39.47%)
Urine	10	6 (69%)	4 (40.0%)
Cx.Swab	14	14 (100%)	0 (0%)
Semen	1	1 (100%)	0 (0%)
Conj.Swab	14	7 (50 %)	7 (50%)
Ear Swab	3	2 (66.66%)	1 (33.33%)
ThroatSwab	3	2 (66.66%)	1 (33.33%)
Stool	7	7 (100%)	0 (0%)
Vomitus	6	6 (100%)	0 (0%)
Body Fluid	3	3 (100%)	0 (0%)
Drin Tip	2	1 (50%0	1 (50%)
CSF	1	0 (0%)	1 (100%)
Total	102	72 (70.58%)	30 (29.41%)

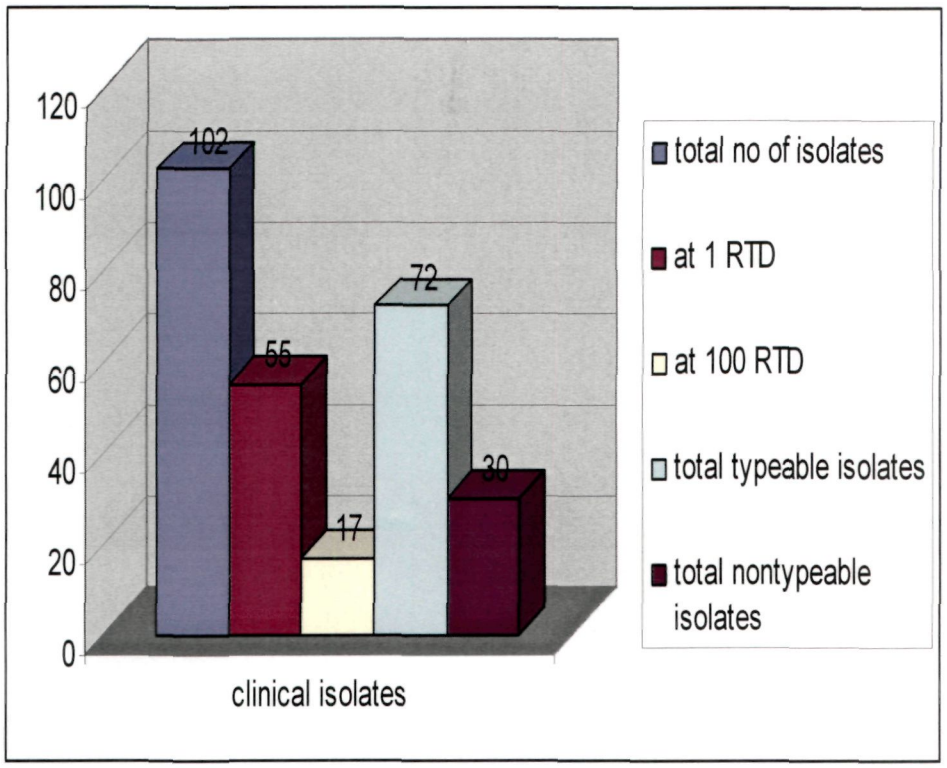


Fig- 4. Bar diagram showing bacteriophage typing at RTD and 100 RTD from various human clinical isolates

(ii) MRSA phage typing of human clinical isolates:

MRSA phage groups were used to type methicillin resistant *Staphylococcus aureus* isolates of human clinical specimens. A total of 14 (41.18%), out of 34 MRSA, were found typeable at 1 RTD and 100 RTD using MRSA phages. Briefly, at 1 RTD out of 15 pus isolates, 3 (20.0%) were typeable and belonged to mixed group; 4 (100%) from Cx. swab were found typeable (one in group III and 3 in mixed group); and 1 (100%) from drain tips (group I). Whereas all isolates from urine, conj. swabs, ear swabs, throat swabs, stool, vomitus, body fluid and CSF were untypeable at 1 RTD (Table - 8).

Table- 8. Bacteriophage typing of methicillin resistant *S. aureus* isolates from human clinical specimens using MRSA phages at 1 RTD

Source	Total No. of Isolates Tested	1 RTD					
		Typeable Groups				Mixed Group	Typeable Group
		I	II	III	Non allocated		
Pus	15					I & III = 3	3
Urine	4						0
Cx. Swab	4			1		1 & 2 = 3	4
Semen	0						0
Conj.Swab	4						4
Ear Swab	1						0
ThroatSwab	1						0
Stool	1						0
Vomitus	1						0
Body Fluid	1						0
Drain tip	1	1					0
CSF	1						0
Total	34	1(2.91%)		1(2.91%)		6(17.71%)	8(27.5%)

However at 100 RTD two pus isolates, out of 12, were typeable in mixed group; 3 urine isolates (one belonged to group II and the other to group III), one vomitus (group III) were found typeable, whereas rest could not be typed (Table - 9).

Table- 9. Bacteriophage typing of methicillin resistant *S. aureus* isolates from human clinical specimens at 100 RTD

Source	Total no. of isolates tested	100 RTD					
		Typeable groups				Mixed group	Typeable group
		I	II	III	Non allocated		
Pus	12					I & III = 2	2
Urine	4		1	2			3
Cx. swab	0						0
Semen	0						0
Conj. swab	4						0
Ear swab	1						0
Throat Swab	1						0
Stool	1						0
Vomitus	1			1			1
Body Fluid	1						0
Drain tip	0						0
CSF	1						0
Total	26		1(3.8%)	3(11.5%)		2(7.7%)	6(23.1%)

The MRSA obtained from various human clinical samples and their combined bacteriophage typing results (at 1 RTD + 100 RTD) are shown in table- 10 and fig- 5.

Table-10. Human clinical isolates of methicillin resistant *Staphylococcus aureus* in relation to combined bacteriophage typing at 1 RTD and 100 RTD

Source	Total no. of isolates tested	Typeable isolates (%)	Nontypeable isolates (%)
Pus	15	5 (33.33)	10 (66.77)
Urine	4	3 (75.0)	1 (25)
Cx.Swab	4	4 (100)	0 (0)
Semen	0	0 (0)	0 (0)
Conj.Swab	4	0 (0)	4 (100)
Ear Swab	1	0 (0)	1 (100)
ThroatSwab	1	0 (0)	1 (100)
Stool	1	0 (0)	1 (100)
Vomitus	1	1 (100)	0 (0)
Body Fluid	1	0 (0)	1 (100)
Drin Tip	1	1 (100)	0 (0)
CSF	1	0 (0)	1 (100)
Total	34	14 (41.18)	20 (58.82)

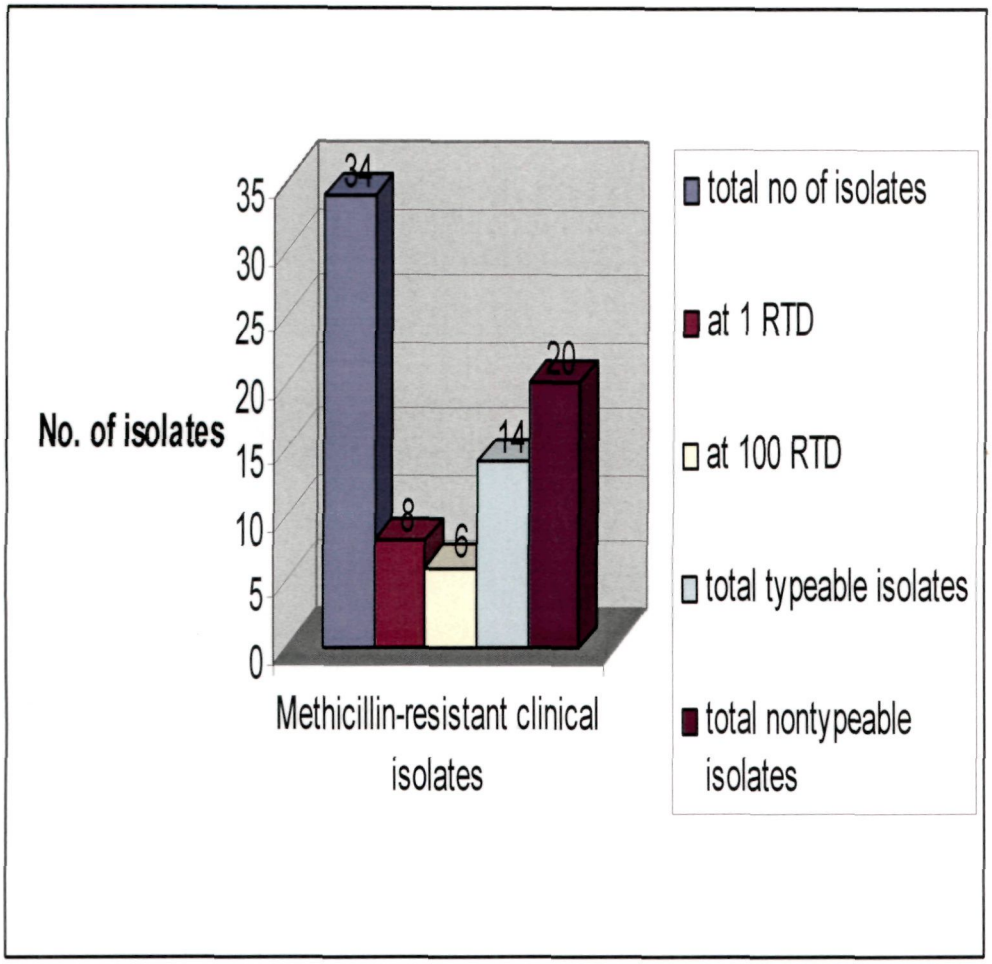


Fig- 5. Bar diagram showing MRSA phage typing at 1RTD and 100 RTD from various human clinical specimens

(iii) Routine bacteriophage typing of animal-origin isolates:

Out of 100 **animal-origin isolates** none of the isolate was found typeable at routine test dilution and 100 RTD.

(iv) MRSA phage typing of animal-origin isolates:

MRSA phage groups at routine test dilution were used to type methicillin-resistant *Staphylococcus aureus* isolates of animal-origin samples. None of the isolate was found typeable at 1 RTD. These 26 isolates were further tested at 100 RTD. Out of which only two isolates (one from animal clinical sample (raw milk) and the other from buffalo

meat) could be typed which belonged to phage group II at 100 RTD (Table - 11) (fig- 6).

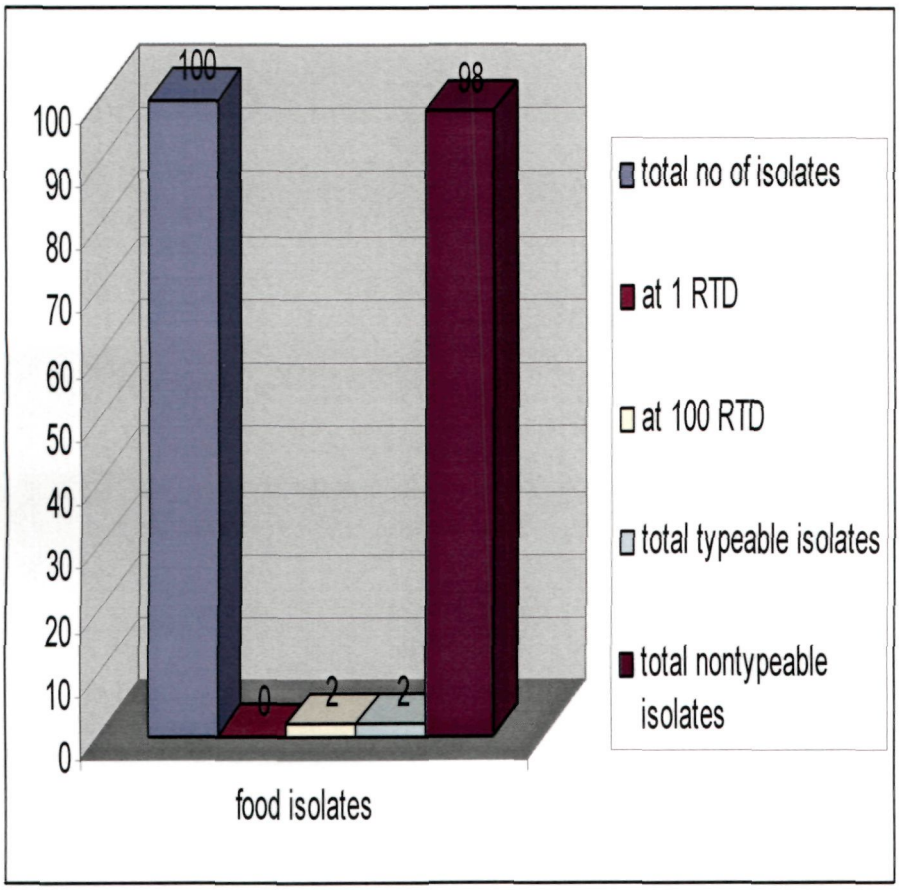
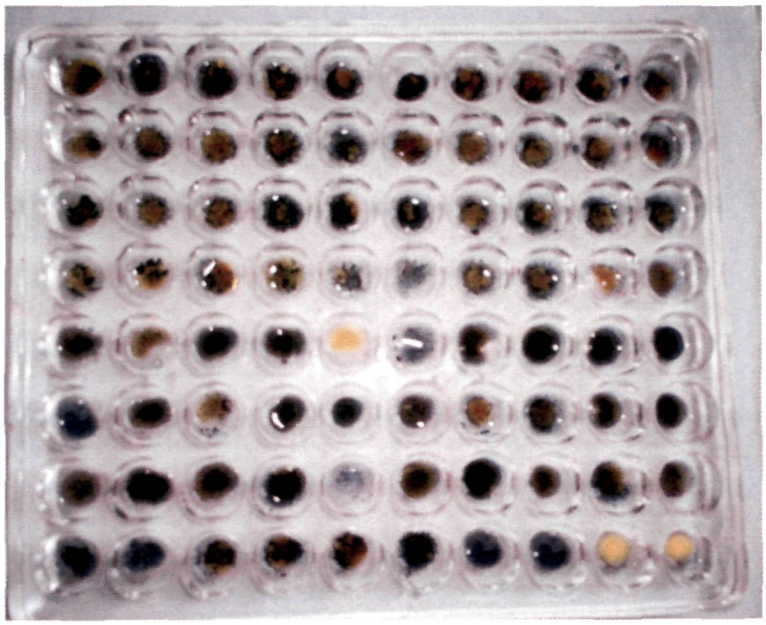


Fig- 6. Bar diagram showing MRSA phage typing at 1RTD and 100 RTD from various animal-origin samples

4.2.3. β - lactamase production:

Out of 102 **human clinical isolates** 80 (78.4%) were β-lactamase producers whereas of 100 **animal-origin isolates** 69 (69%) were found β-lactamase producers by iodometric method (fig- 7).



Negative control Positive control

Fig - 7 Photograph showing Beta lactamase test by Idometric method

Table- 11. Bacteriophage typing of *S. aureus* isolates from animal-origin food samples

Source	Total no. of isolate	RTD						100x RTD					
		Typeable group				Mixed group	Nontypeable group	Typeable group				Mixed group	Nontypeable group
		I	II	III	N. A.			I	II	III	N. A.		
Animal clinical specimen (raw milk)	29						29		1				28
Khoa	9						9						9
Cottage Cheese	6						6						6
Chamcham	4						4						4
Sweet	3						3						3
Goat meat	27						27						27
Buffallo meat	20						20		1				19
Kabab	1						1						1
Salami	1						1						1
Total	100						100		2				98

4.3. Genotypic Characterization:

The preparation of templates for PCR assay was done by the following methods,

1. Genomic DNA extraction: by phenol chloroform extraction method
2. Bacterial cell lysates: by using
   a) Triton X – 100
   b) SDS
   c) Sonication
   d) Boiling and Chilling

Out of these template preparation methods, the template prepared by phenol chloroform extraction method gave clear-cut amplification. Heat lysis method (Boiling and Chilling) also gave same amplification equivalent to that of phenol chloroform extraction. However, template prepared by triton X-100 method, SDS, and sonication method did not yield any PCR product. As the conventional phenol chloroform extraction method is time consuming, the boiling and chilling method was used in subsequent genotypic studies.

4.3.1. Results of PCR for *coa* gene:

The detection of the ***coa*** gene was done by Polymerase Chain Reaction using the primer set described by Goh *et al.* (1992).

4.3.1.1. PCR for *coa* gene of human clinical isolates:

C*oa* gene could be amplified in all the **human clinical isolates.** Twelve different electrophoretic patterns were observed in these isolates. Thirty strains (29.4%) showed a distinctive bands of 500 bp and 590 bp, 22 strains (21.6%) showed bands of 480 bp, 680 bp and 800 bp, 10 strains (9.8%) showed band of 500 bp and 580 bp, 8 strains (7.8%) showed bands of 500 bp, 720 bp and 800 bp, 7 strains (6.9%) showed bands of 500 bp, 580 bp, 700 bp and 800 bp, other 7 strains (6.9%) showed bands of 580 bp, 700 bp and 800 bp, where as other 5 strains (4.9%) showed bands of 480 bp, 500 bp and 580 bp, other 4 strains (3.9%) showed bands of 550 bp, 780 bp and 900 bp, 3 strains each (2.9)% showed 500 bp, 580 bp and 720 bp: and 550 bp, 800 bp and 900 bp respectively, 2 strains (1.9%) showed bands of 470 bp, 700 bp, 790 bp and 920 bp, whereas 1 strains (1.0%) showed bands of 550 bp, 600 bp and 820 bp, (fig- 8). Maximum numbers of strains showed a band pattern of 500 bp and 580 bp and were obtained from pus isolates.

4.3.1.2. PCR for *coa* gene of animal-origin isolates:

In 100 **animal-origin isolates** 7 electrophoretic patterns were observed. Forty seven strains (47%) showed a band pattern of 900 bp, 17 strains (17%) showed bands of 650 bp and 700 bp, 13 strains (13%) showed bands of 590 bp, 680 bp and 700 bp, 8 strains (8%) showed bands of 600 bp, 7 strains (7%) showed bands of 650 bp, other 7 strains (7%) showed bands of 650 bp and 750 bp, whereas 1 strain (1%) showed bands of 500 bp and 820 bp (fig- 9).

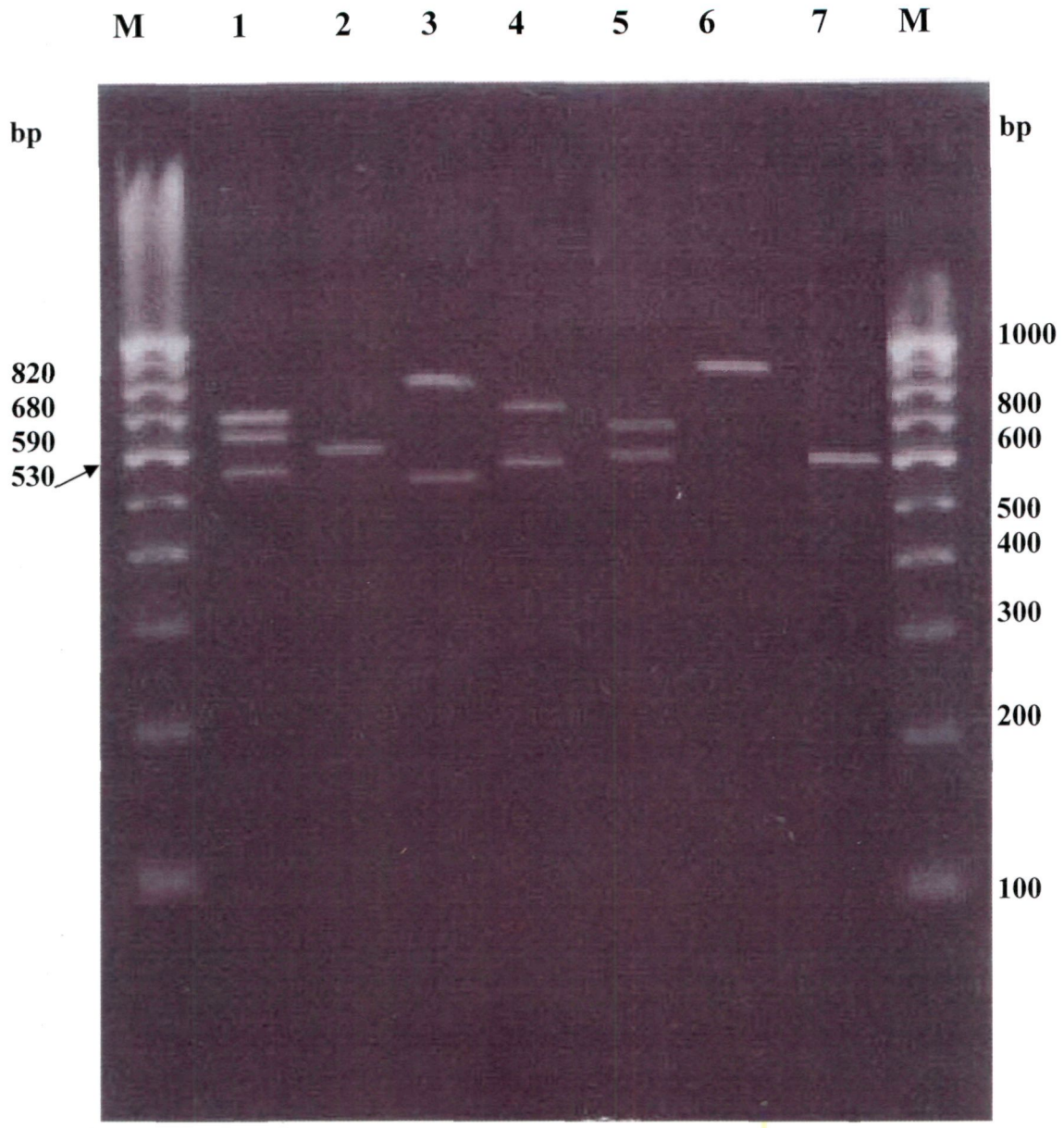


Fig-9 Electrophoretogram showing coagulase gene patterns of animal-origin food samples run on 2% agarosegel. Lane M shows molecular weight marker (100bp). Lanes 1to 7 shows groups 1-7 respectively

4.3.2. Results of *coa* – RFLP:

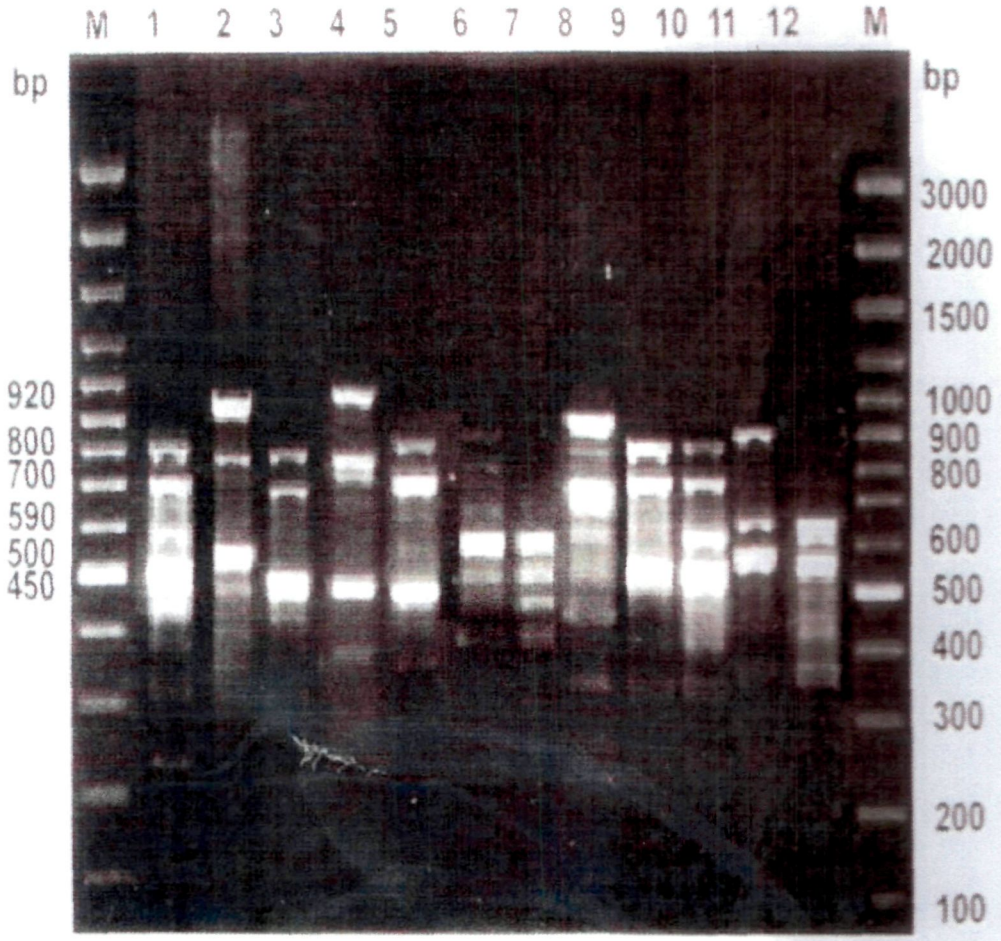
The molecular typing of the ***coa*** gene of the 202 (102 human clinical isolates and 100 animal-origin isolates) isolates was carried out by primer-specific Polymerase Chain Reaction (PCR), after the digestion with *Alu 1* as described by Goh *et.al.*, 1992.

4.3.2.1. Results of *coa* – RFLP of human clinical isolates:

A total 12 types of ***coa*** **-RFLP** patterns were observed in **human clinical isolates**. Majority 30 (29.4%) of strains showed bands of 243 bp and 405 bp were classified in group XII followed by 22 strains (21.6%) in group III showing bands of 81 bp, 243 bp while 10 strains (9.8%) were characterized by distinctive bands pattern of 162 bp and 243 bp and were characterized in group VI. Eight strains (7.8%) having bands of 405 bp and 567 bp were categorized in group IX. Seven strains (6.9%) each showed bands of 81bp, 162 bp, 243 bp, 324 bp, 405 bp and 162 bp, 324 bp, 405 bp, respectively, and were classified in group I and group VIII. Five strains (4.9%) and 4 strains (3.9%) each showed bands of 81 bp, 162 bp: and 162 bp and 324 bp, respectively, were classified in group VII and group II, 3 strains (2.9%) each were classified in group IV and group X consisting of band patterns of 162 bp, 243 bp, 324 bp, 567 bp and 162 bp, 332 bp, 405 bp, respectively. Two strains (1.9%) showed band patterns of 162 bp, 243 bp, 405 bp belonged to group XI. However, 1 strain (1.0%) of group V showed band pattern of 243 bp, 324 bp (fig- 10). Heterogeneity was observed in specimens of a similar type. For example, *S. aureus* isolates from cervical swabs belonged to six different coa-RFLP (Table-12).

Table- 12. Pattern of coagulase gene restriction fragment length polymorphism of *S. aureus* isolates from clinical specimens

Specimens	No. of isolates tested	Coa-RFLP Groups											
		I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Pus	38	3	0	8	0	1	8	1	3	2	0	0	10
Urine	10	2	1	1	1	0	0	0	0	0	0	0	5
Cx. Swab	14	1	0	4	0	0	0	0	2	0	0	1	6
Semen	1	0	0	0	0	0	0	0	0	0	0	1	0
Conj. Swab	14	1	1	1	0	0	1	2	1	3	0	0	4
Ear Swab	3	0	0	0	1	0	0	0	0	0	0	0	2
Throat Swab	3	0	0	0	1	0	0	2	0	0	0	0	0
Vomitus	6	0	0	1	0	0	0	0	1	3	1	0	1
Stool	7	0	1	5	0	0	0	0	0	0	0	0	1
CSF	1	0	0	0	0	0	0	0	0	0	0	0	1
Drain Tip	2	0	1	0	0	0	0	0	0	0	1	0	0
Body Fluid	3	0	0	2	0	0	1	0	0	0	1	0	0
Total	102	7	4	22	3	1	10	5	7	8	3	2	30



Electrophoretogram showing coagulase gene pattern of clinical specimens run on 1.5% agarosegel. Lane M shows molecular weight marker (100bp). Lane 1 to 12 shows groups 1-12 respectively

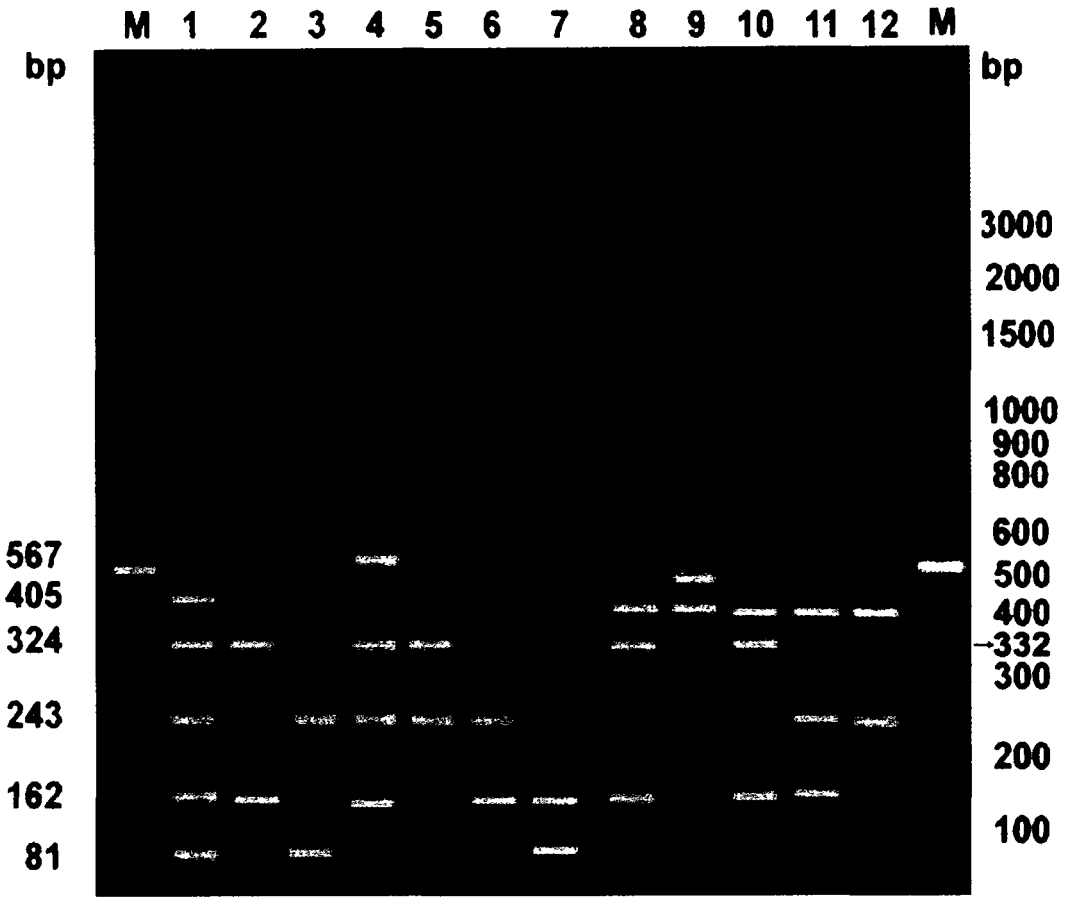


Fig-1 Electrophoretogram showing coa-RFLP gene pattern of clinical specimens run on 2% agrosegel. Lane M shows molecular weight marker (100 bp). Lane 1-12 shows groups 1-12 respectively

(i) *coa* -RFLP patterns in MRSA and MSSA:

Among these 12 patterns, 7 groups patterns, namely, group I, group II, group IV, group V, group VI, group VIII and group XI were noticed in MRSA isolates. Rest of the group patterns were in MSSA isolates.

(ii) *coa* -RFLP patterns of MRSA in relation to antibiotic resistance patterns: Out of 34 methicillin resistant *Staphylococcus aureus* isolates from human clinical specimens 7 (20.6%) belonged to *Coa*-RFLP pattern of group I where majority of strains were resistant to six or more antibiotics; 10 strains (29.4%) belonged to *Coa*-RFLP group VI and 4 strains (11.8%) of *Coa*-RFLP group II were also found resistant to six or more antibiotics. While 7 (20.6%) strains belonged to *Coa*- RFLP group VIII having resistance to five or more antibiotics. *Coa*-RFLP group IV and group XI having 3 strains (8.8%) and 2 (5.9%) strains, respectively, also showed resistance to five or more antibiotics, however, only one strain (2.9%) which belonged to *Coa*-RFLP group V showed resistance to five antibiotics (Table - 13).

Table – 13. Resistance patterns in human clinical MRSA isolates in relation to *Coa*-RFLP groups

Coa-RFLP groups	Total no. of isolates (%)	Resistance patterns (n= no. of isolates)
Group I	7 (20.58%)	P E T G C Cf Co Ca (2) P E T C Cf CoCa (4) P E T G Cf Co Ca (1)
Group II	4 (11.76%)	P E T C Co Ca (2) P E T G Cf Ca (2)
Group IV	3 (8.82%)	P T G Cf CoCa (2) P C Cf Co Ca (1)
Group V	1 (2.94%)	P E T Cf Ca (1)
Group VI	10 (29.41%)	P E T G C f Co Ca (5) P E T Cf Co Ca (5)
Group VIII	7 (20.59%)	P E T G C Cf Ca (1) P E T C Cf Ca (1) P E T C Cf Co Ca (1) P T C Cf Co Ca (1) P G Cf Cc Ca (2) P C Cf Co Ca (1)
Group XI	2 (5.88%)	P G Cf Co Ca (1) E T Cf Co Ca (1)

Abbreviation Key:

P = Penicillin G, A = Ampicllin, Ak = Amikacin, Ac = Amoxycillin, C = Chloramphenicol, Cf = Ciprofloxacin, Co = Co –Trimoxazole, Cj = Cefaclor, Ci = Ceftriaxone, Cpm = Cefepime, Ca = Ceftazidime, E = Erythromycin, T = Tetracycline, G = Gentamicin, Ox = Oxacillin

Among these 12 patterns, 5groups patterns, namely, group III, group VII, group IX, group X, and group XII were noticed in MSSA isolates.

Antibiotic resistance patterns in relation to *Coa* - RFLP pattern could not be inferred in isolates obtained from clinical specimens due to variability of resistance patterns; 40 different types of patterns were observed.

4.3.2.2. Results of *coa* – RFLP of animal-origin isolates:

In 100 **animal-origin isolates,** 7 types of Coa-RFLP patterns were observed, majority (47%) were classified in group VI with band of 405 bp followed by 17% strains characterized by distinctive band patterns of 243bp and 324 bp in group V, 13% strains in group I consisting of 162 bp, 250 bp and 405 bp, 8% strains showed bands of 81 bp and 324 bp belonged to group VII, and 7% strains showed bands of 324 bp and were characterized as group II, 3% strains of group IV showed band patterns of 162 bp and 405 bp, 1 (1%) strain of group III showed band pattern of 162 bp and 324 bp. Five out of 29 animal-origin clinical isolates (raw milk) were classified in group VII and group II with band patterns of 81 bp, 324 bp and 324 bp (Table-14) (Fig-11). Mixed Coa-RFLP gene patterns were observed in animal clinical specimens and animal-origin food samples. To appreciate the reproducibility of coa-RFLP the test was performed in duplicate. The reproducibility was good as similar results were obtained in both the tests.

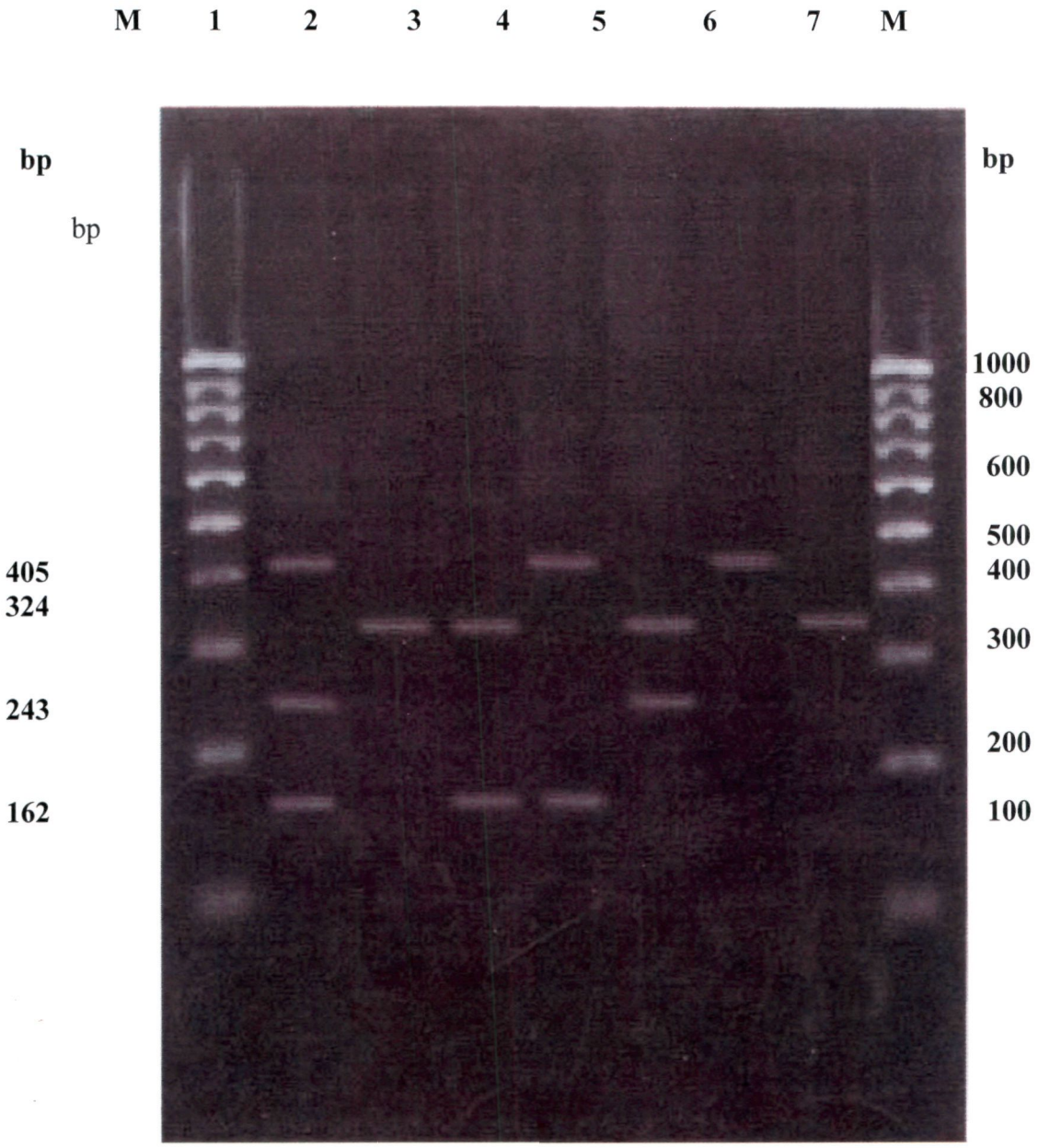


Fig-11 Electrophoretogram showing Coa-RFLP gene patterns of animal-origin food samples run on 2% agarosegel. Lane M shows molecular weight marker (100bp). Lanes 1to 7 shows groups 1-7 respectively

Table- 14. Coagulase gene restriction fragment length polymorphism of *S. aureus* isolates from animal-origin samples

Specimens	No. of Isolate tested	Coa- RFLP-Groups						
		I	II	III	IV	V	VI	VII
Animal clinical specimen (Raw milk)	29	4	5	0	3	4	8	5
Chamcham	4	1	0	0	0	0	2	1
Sweet	3	1	0	1	0	0	1	0
Khoa	9	2	0	0	1	2	4	0
Paneer	6	2	1	0	0	1	2	0
Goat Meat	27	2	1	0	3	6	15	0
Buffalo Meat	20	0	0	0	0	4	14	2
Kabab	1	1	0	0	0	0	0	0
Salami	1	0	0	0	0	0	1	0
Total	100	13	7	1	7	17	47	8

(i) *coa* -RFLP patterns in MRSA and MSSA:

Among these 7 patterns, 4 group patterns, namely, group II, group III, group I, and group VII, were noticed in 29 MRSA isolates from animal-origin samples. Rest of the group patterns were in MSSA isolates.

(ii) *coa* -RFLP patterns in relation to antibiotic resistance patterns: Out of 29 methicillin resistant *Staphylococcus aureus* isolates from

animal-origin samples 8 (27.6%) belonged to *Coa*-RFLP pattern of group VII where majority of strains were resistant to six antibiotics. While 13 (44.8%) strains belonged to *Coa*- RFLP group I having resistance to five antibiotics. *Coa*-RFLP group II having resistance to seven or more antibiotics was noticed in 7 strains (24.1%), however, only one strain (3.4%) showed resistance to three antibiotics and belonged to *Coa*-RFLP group III (Table - 15).

Table – 15. Resistance patterns in animal-origin MRSA isolates in relation to *Coa*-RFLP groups

Coa-RFLP groups	Total no. of isolates (%)	Resistance patterns (n= no. of isolates)
Group I	13 (44.82%)	E T Cf Co Ca (1)
		P E T Co Ca (1)
		E T C Cf Ca (1)
		E C Co Ca (2)
		P E T C Ca (2)
		E C Co Ca (2)
		T G Co Ca (2)
		P E T Ca (2)
Group II	7 (24.14%)	P E T G C Cf Co Ca (3)
		P E T C Cf Co Ca (1)
		P E T G Cf Co Ca (2)
		P T G Cf Co Ca (1)
Group III	1 (3.45%)	P T Ca (1)
Group VII	8 (27.59%)	P E T G C Ca (2)
		P E T C Co Ca (2)
		P E T,G Co Ca (4)

Abbreviation Key:
P = Penicillin G, C = Chloramphenicol, Cf = Ciprofloxacin, Co = Co –Trimoxazole, Ca = Ceftazidime, E = Erythromycin, T = Tetracycline, G = Gentamicin,

Among the 7 patterns noticed in isolates from animal-origin samples, 3 group patterns, namely, group IV, group V, and group VI were noticed in MSSA isolates. Antibiotic resistance patterns in relation to *Coa* – RFLP pattern could not be inferred in remaining isolates obtained from animal-origin samples due to variability of resistance patterns; 40 different types of patterns.

4.3.3. Results of PCR for Thermostable Nuclease Gene (*nuc*):

Two hundred and two coagulase positive *staphylococcus aureus* isolates including 102 human clinical and 100 animal-origin isolates were tested for the presence of thermostable nuclease *(nuc)* gene by Polymerase Chain Reaction using the method of Brakstad *et al.* (1992) with slight modification. All the 202 isolates showed a 270 bp amplified product after gel electrophoresis which is specific for the presence of *nuc* gene in *staphylococcus aureus* (fig- 12).

4.3.4. Results of PCR for Enterotoxin Genes (SEA, SEB and SEC):

For the detection of enterotoxin genes, namely enterotoxin A, enterotoxin B and enterotoxin C, PCR assay was used to amplify specific base pair products. A total of 202 isolates (102 human clinical isolates and 100 animal-origin isolates) were tested for production of enterotoxin A, enterotoxin B and enterotoxin C. For the enterotoxin A and enterotoxin B modified method of Johnson *et al.* (1991) was used, which gave 120 bp (fig- 13) and 478 bp (fig- 14) gene specific products, respectively, while the modified method of Chen *et al.* (2001) was used to amplify enterotoxin gene C which showed a gene specific 234 bp (fig- 15) product.

Of the 102 human clinical isolates 3 were found enterotoxogenic, out of which 1 from stool sample were found

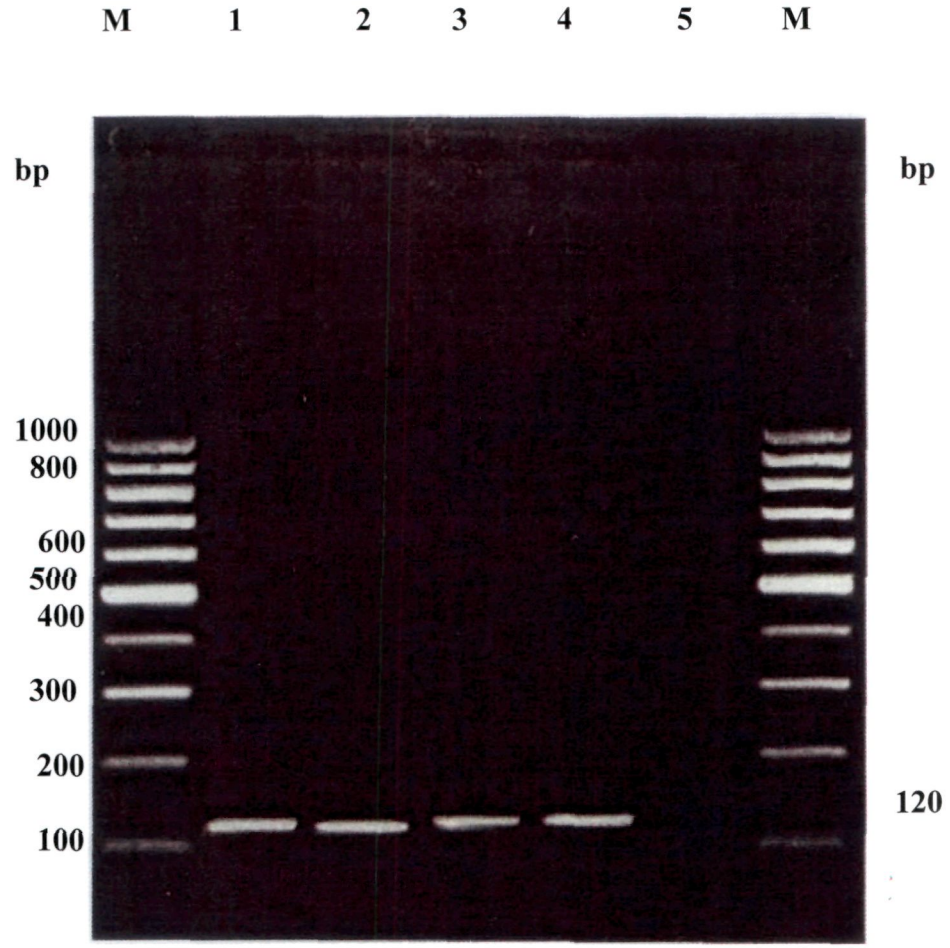


Fig-13 Electrophoretogram showing 120 bp amplicon of SEA gene on 1.5% agarosegel. Lane M shows100bp ladder. Lanes 1 to 4 shows positive samples. Lane 5 shows negative sample.

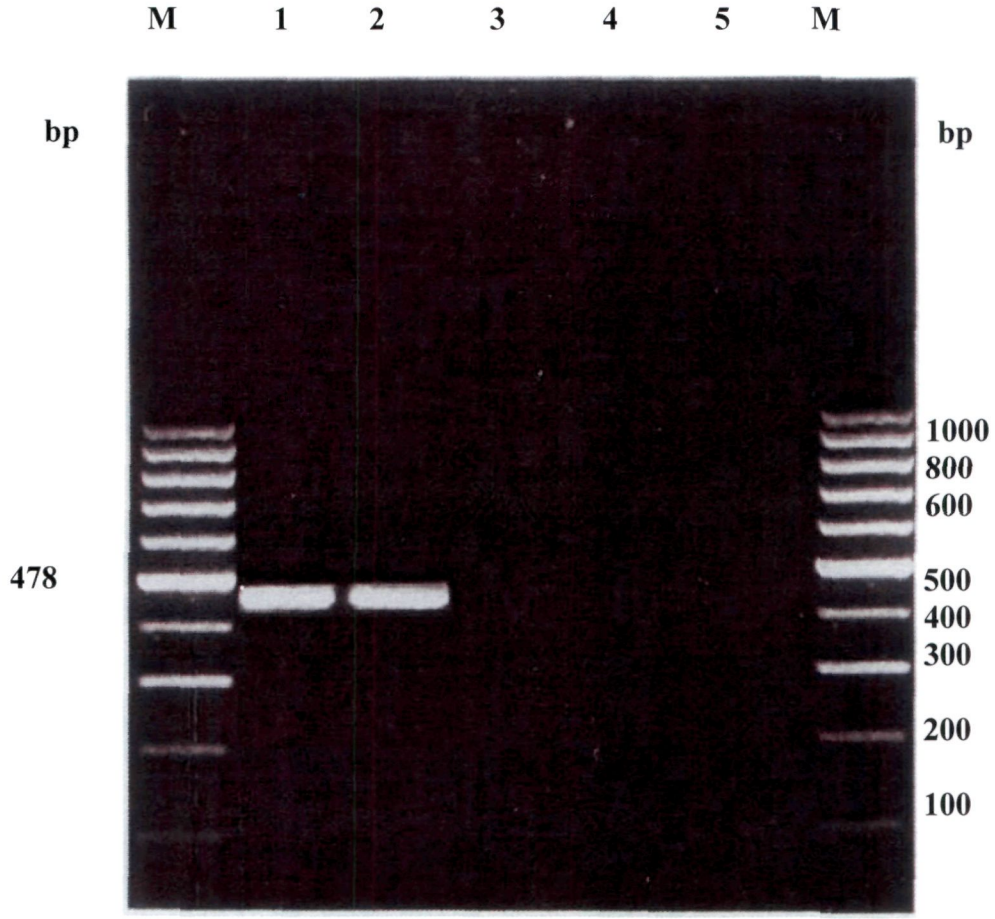


Fig-14 Electrophoretogram showing 478 bp amplicon of SEB on 1.5% agarosegel. Lane M shows 100bp ladder. Lanes 1 to 2 shows positive samples. Lanes 3-5 shows negative samples.

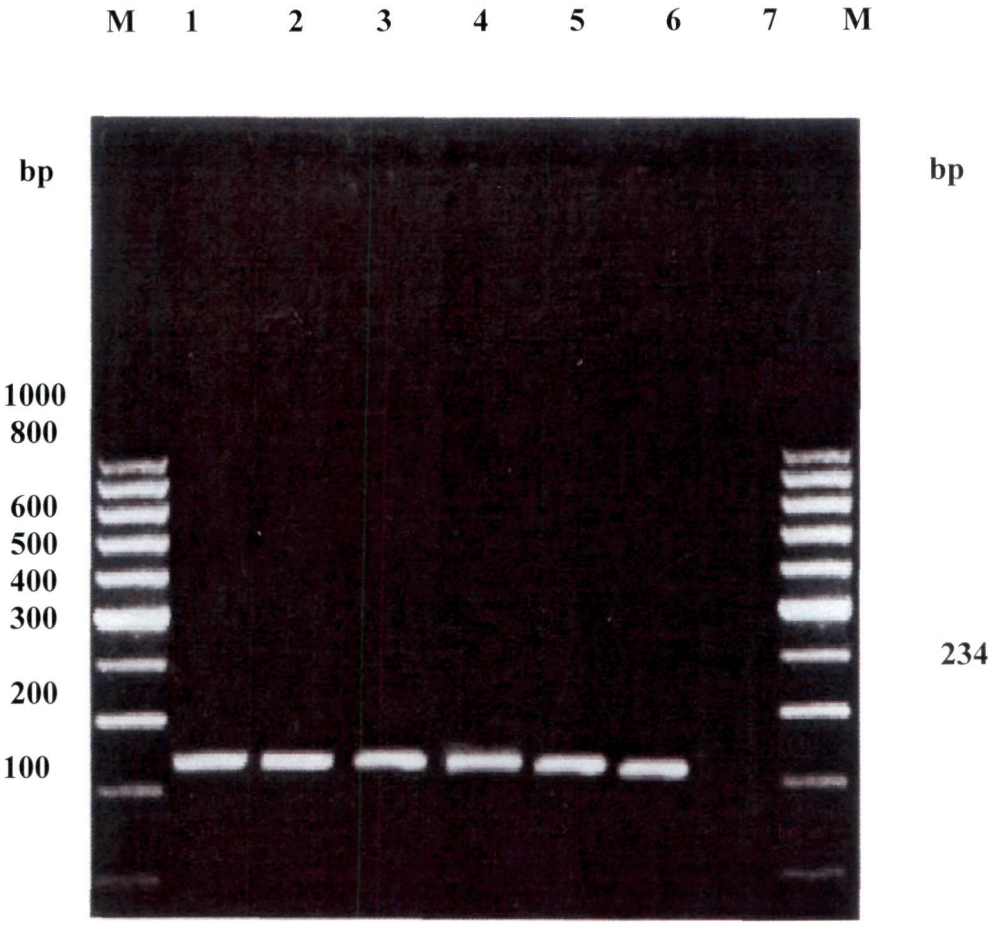


Fig-15 Electrophoretogram showing 234 bp amplicon of SEC gene on 1.5% agarosegel. LaneM shows 100bp ladder. Lanes 1 to 6 shows positive samples. Lane 7 shows negative sample.

positive for enterotoxin A, 1 from vomitus showed presence of enterotoxin B while the other 1 from urine sample shows presence for enterotoxin C (Table - 16).

Table- 16. Prevalence of staphylococca enterotoxin genes in *Staphylococcus aureus* isolates from clinical specimens

Specimens	No. of Isolates tested	No. of samples positive for enterotoxins			Total
		SEA	SEB	SEC	
Pus	38				0
Urine	10			1	1
Cx. Swab	14				0
Conj. Swab	14				0
Ear Swab	3				0
ThroatSwab	3		0		0
Semen	1				0
Stool	7	1			1
Vomitus	6		1		1
Body Fluid	3				0
Drain tip	2				0
CSF	1				0
Total	102	1	1	1	3

Out of 100 **animal-origin isolates** a total of 13 (13%) were found enterotoxigenic. Of these 2 were positive for enterotoxin A (2%) (1 from

clinical specimens of animal (raw milk) s mple and 1 from buffalo meat); 3 (3%) were positive for enterotoxin B (1 from clinical specimens of animal (raw milk) sample, 1 from sweet and 1 from cottage cheese) and 8 (8%) were found positive for enterotoxin C (1 from clinical specimens of animal (raw milk), 1 from paneer, 3 from goat meat and 3 from buffalo meat) (Table - 17).

Table- 17. Prevalence of staphylococcal enterotoxin genes in *Staphylococcus aureus* isolates from animal-origin samples

Specimens	No. of Isolates tested	No. of samples positive for enterotoxins			Total
		SEA	SEB	SEC	
Animal origin clinical specimens (Raw Milk)	29	1*	1*	1*	3
Chamcham	4				0
Sweet	3		1		1
Khoa	9				0
Cottagecheese	6		1	1	2
Goat Meat	27			3	3
Buffalo Meat	20	1*		3*	4
Kabab	1				0
Salami	1				0
Total	100	2	3	8	13

*Enterotoxins (SEs) were detected from different samples.

Antibiotic resistance patterns of enterotoxigenic strains:

Among the 16 enterotoxigenic *Staphylococcus aureus* isolates, 13 different antibiotic resistance patterns were observed. All of the enterotoxigenic isolates from human clinical specimen showed resistance to methicillin. However all of the strains exhibited different antibiotic resistance pattern. Out of 13 enterotoxigenic animal-origin isolates 10 different antibiotic resistance patterns were observed. Eight of them also showed methicillin resistance. The resistance patterns of enterotoxogenic isolates are shown below in table- 18.

Table- 18. Antibiotic resistance patterns found in enterotoxigenic isolates

Enterotoxin detected (n)		Antibiotic resistance pattern (n)
Ent A (3)	Food (2)	Ac Ak Ox Cj Ci Ca Cpm (1) P E T G Co Ox Cj Ci Ca Cpm (1)
	Clinical (1)	P Ak E T Cf Co Ox Cj Ci Ca Cpm (1)
Ent B (4)	Food (3)	P E T G (1) P T Ak Ox Cj Ci Ca Cpm (2)
	Clinical (1)	P Ak E Cf Co Ox Cj Ci Ca Cpm (1)
Ent C (9)	Food (8)	P E T G Co Cf Ac Ak (2) P E T G Co Ox Cj Ci Ca Cpm (2) P E T G ( ) T E Cf Co Ox Cj Ci Ca Cpm (1) P T Ak Ox Cj Ci Ca Cpm (1) P G E Co (1)
	Clinical (1)	P E T G Cf Ox Cj Ci Ca Cpm (1)

Abbreviation Key:
P = Penicillin, Ak = Amikacin, Ac = Amoxycillin, C = Chloramphenicol, Cf = Ciprofloxacin, Co = Co –Trimoxazole, Cj = Cefaclor, Ci = Ceftriaxone, Cpm = Cefepime, Ca = Ceftazidime, E = Erythromycin, T = Tetracycline, G = Gentamicin, Ox = Oxacillin.

4.3.4. Results of PCR for Methicillin resistant *Staphylococcus aureus* (MRSA):

Methicillin resistant *Staphylococcus aureus* (MRSA) is a major nosocomial pathogen worldwide and is often difficult to detect due to the heterogeneous nature of expression of oxacillin resistance. A total of 102 clinical and 100 animal origin food isolates were tested for oxacillin resistant by disc diffusion method, by screen agar plate and detection of *mec* A gene by PCR. For the detection of *mec* A gene by PCR, primers described by Prasad *et al.* (2000) were used which gave a clear cut specific 604 bp amplified product (fig. 16).

Out of 102 human clinical isolates 34 (33.4%) showed the presence of *mec* A gene. Phenotypically, 33 (32.35%) strains were oxacillin resistant by disc diffusion method and 32 (31.37%) strains showed resistance by screen agar plate method (fig-17).

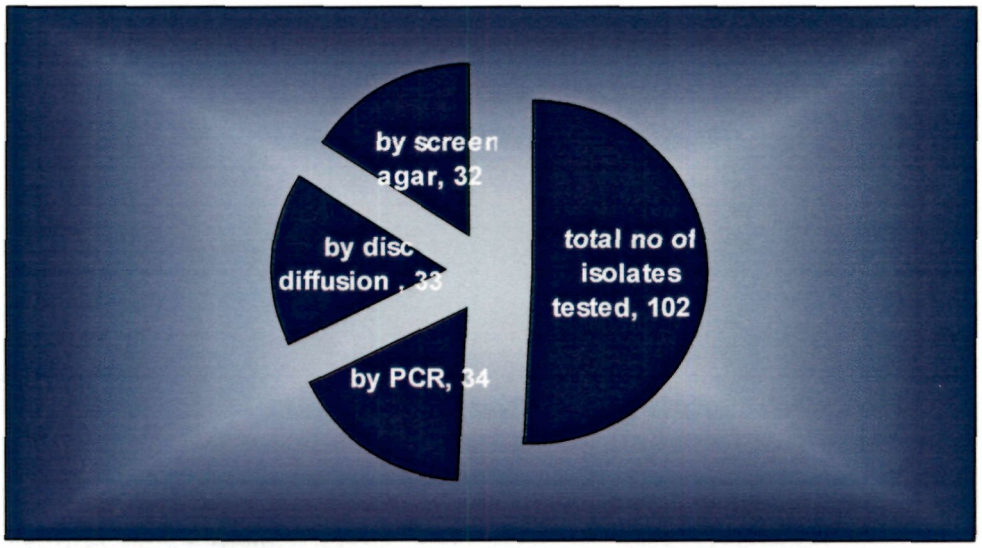


Fig- 17. Contrasting pie chart showing data values of MRSA isolates from human clinical isolates by using various methods

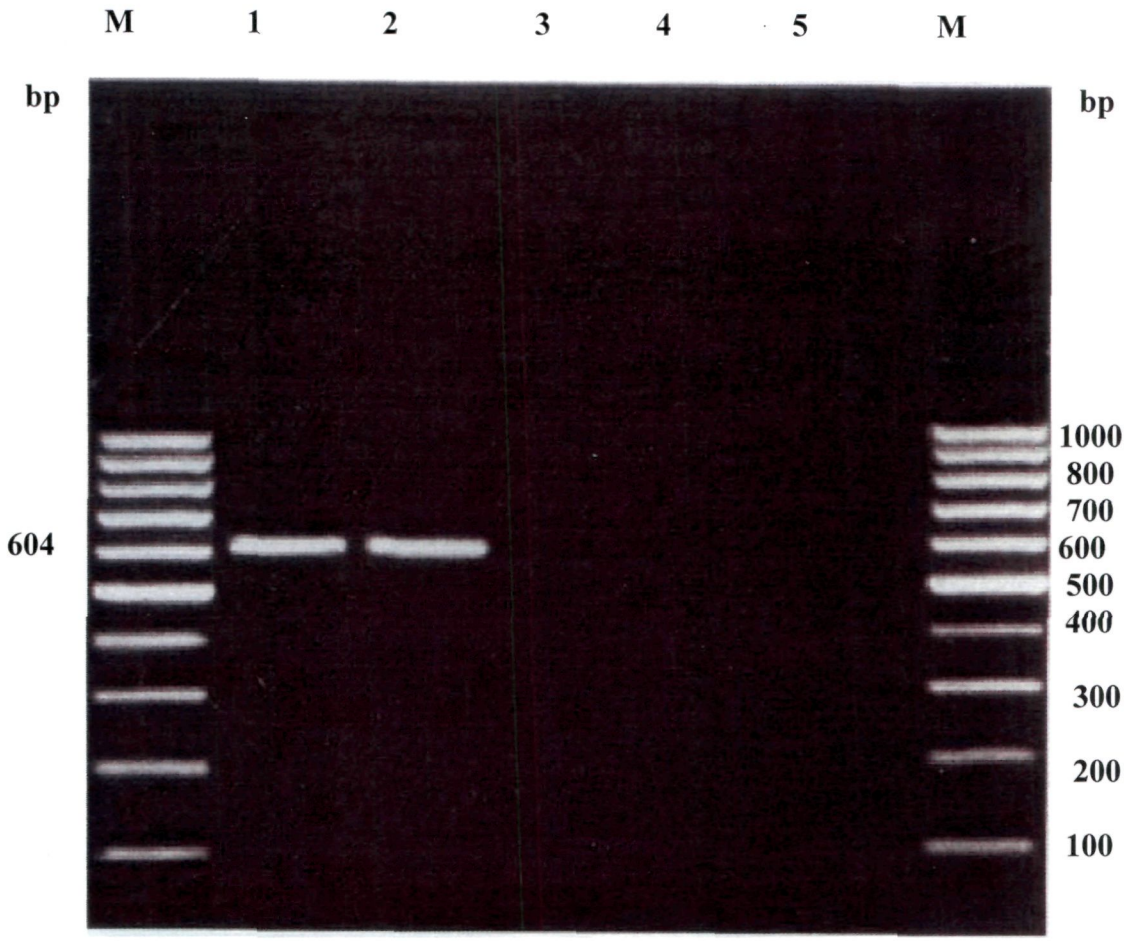


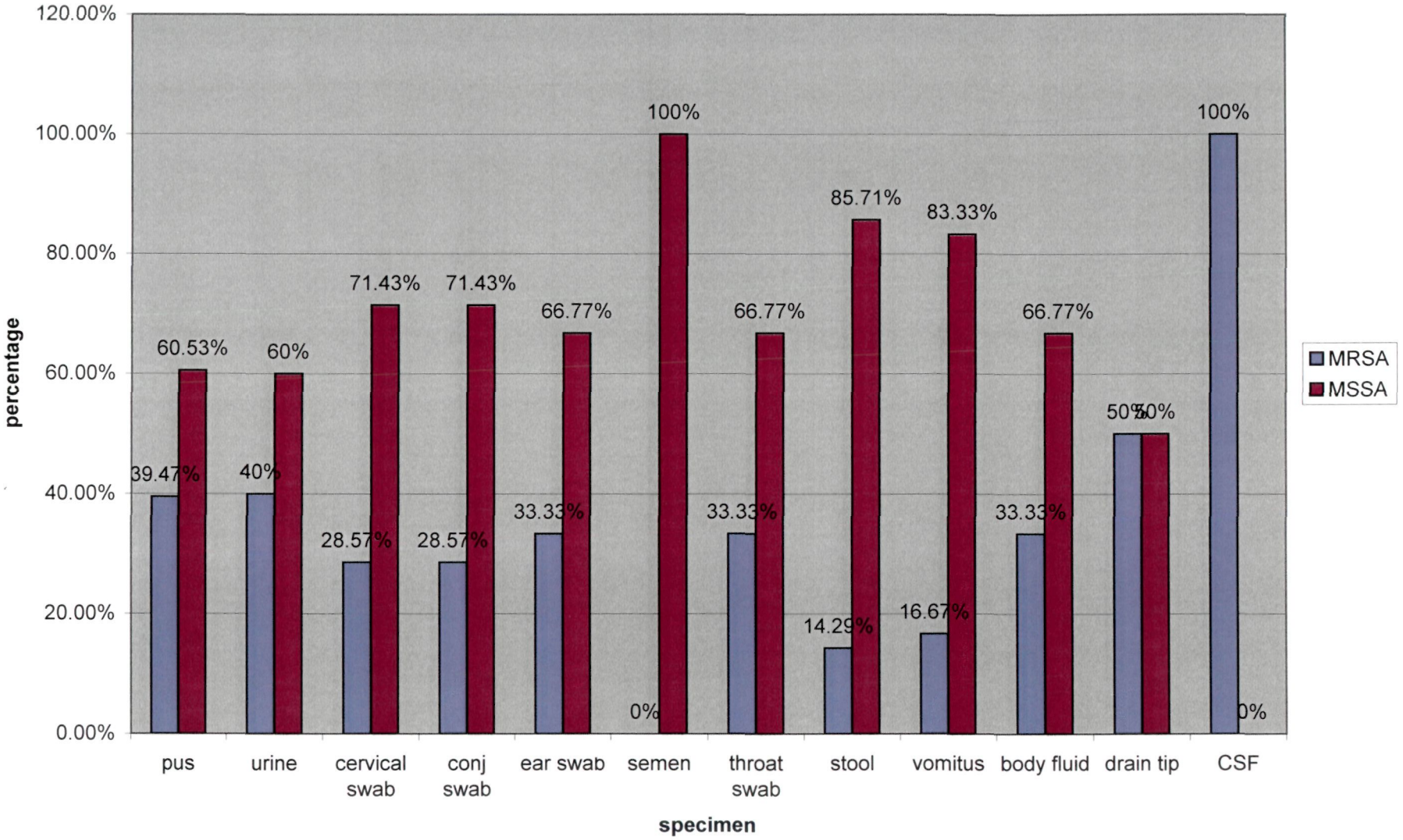
Fig-16 Electrophoretogram showing 604 bp amplicon of MecA gene on 1.5% agarosegel. Lane M shows 100bp ladder. Lanes 1 to 2 shows positive samples. Lanes 3-5 shows negative samples.

Briefly, out of 38 isolates from pus, 39.5% were found positive for *mec* A by PCR, whereas 36.8% showed resistance to oxacillin by disc diffusion and screen agar methods. Among conj. swab and cervical swab, 28.6% isolates each and 40% from urine samples were found resistant by all methods. The comparative detection of methicillin resistance by these three methods in relation to the clinical samples is shown in table -19 (fig. 18).

Table- 19. Detection of Methicillin resistant *Staphylococcus aureus* isolates from human clinical specimens by various diagnostic methods

Specimens	Total no. of isolates	Methicillin resistant isolates detected by		
		Disc Diffusion	Screen Agar	PCR
Pus	38	14 (36 8)	14 (36.8)	15 (39.5)
Urine	10	4 (40.0)	4 (40.0)	4 (40.0)
Cx. Swab	14	4 (28.6)	4 (28.6)	4 (28.6)
Conj. Swab	14	4 (28.6)	4 (28.6)	4 (28.6)
Ear Swab	3	1 (33.3)	1 (33.3)	1 (33.3)
ThroatSwab	3	1 (33.3)	0 (0)	1 (33.3)
Semen	1	0 (0)	0 (0)	0 (0)
Stool	7	1 (14.3)	1 (14.3)	1 (14.3)
Vomitus	6	1 (16.7)	1 (16.7)	1 (16.7)
Body Fluid	3	1 (33.3)	1 (33.3)	1 (33.3)
Drain tip	2	1 (50.0)	1 (50.0)	1 (50.0)
CSF	1	1 (100)	1 (100)	1 (100)
Total	102	33(32.35%)	32(31.37%)	34(33.4%)

Fig- 18 Bar diagram showing methicillin resistance in relation to clinical specimens



Figures in parentheses indicate percentage

In 100 animal-origin isolates, 29 showed the presence of *mec* A gene (fig-16) and of these, 27 strains were oxacillin resistant by disc diffusion method and 26 strains by screen agar plate (fig-19).

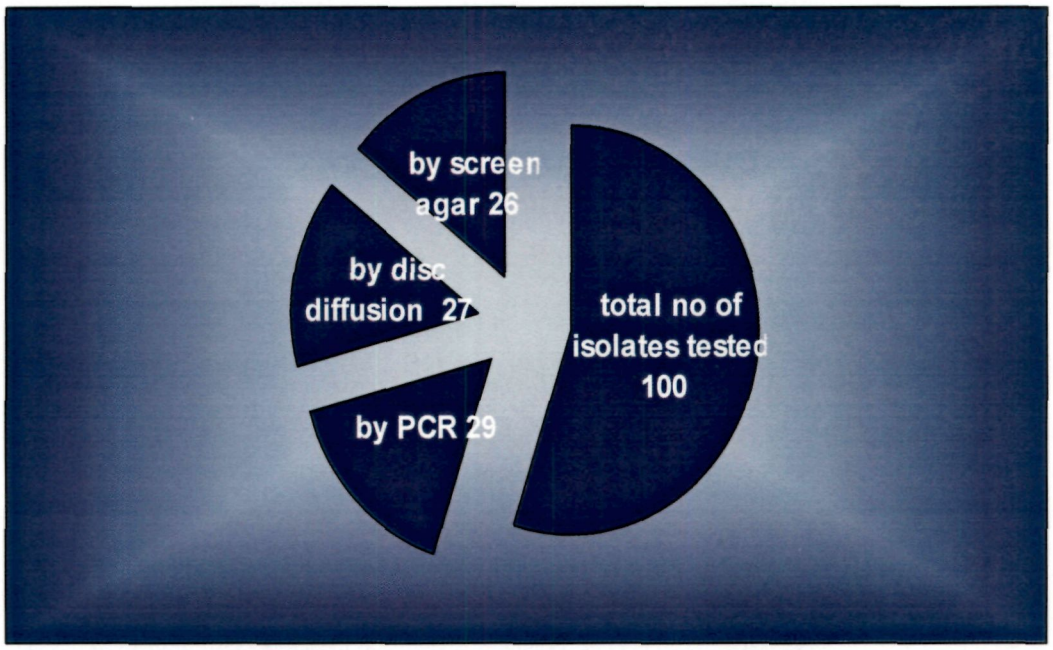


Fig- 19. Contrasting pie chart showing data values of MRSA isolates from animal-origin isolates by using various methods

Briefly, out of 29 samples of animal clinical specimens (raw milk) 15.0% isolates were found methicillin resistant by disc diffusion test, whereas 14.0% isolates showed resistance to oxacillin by screen agar method and presence of *mec* A gene by PCR. In chamcham, sweet,

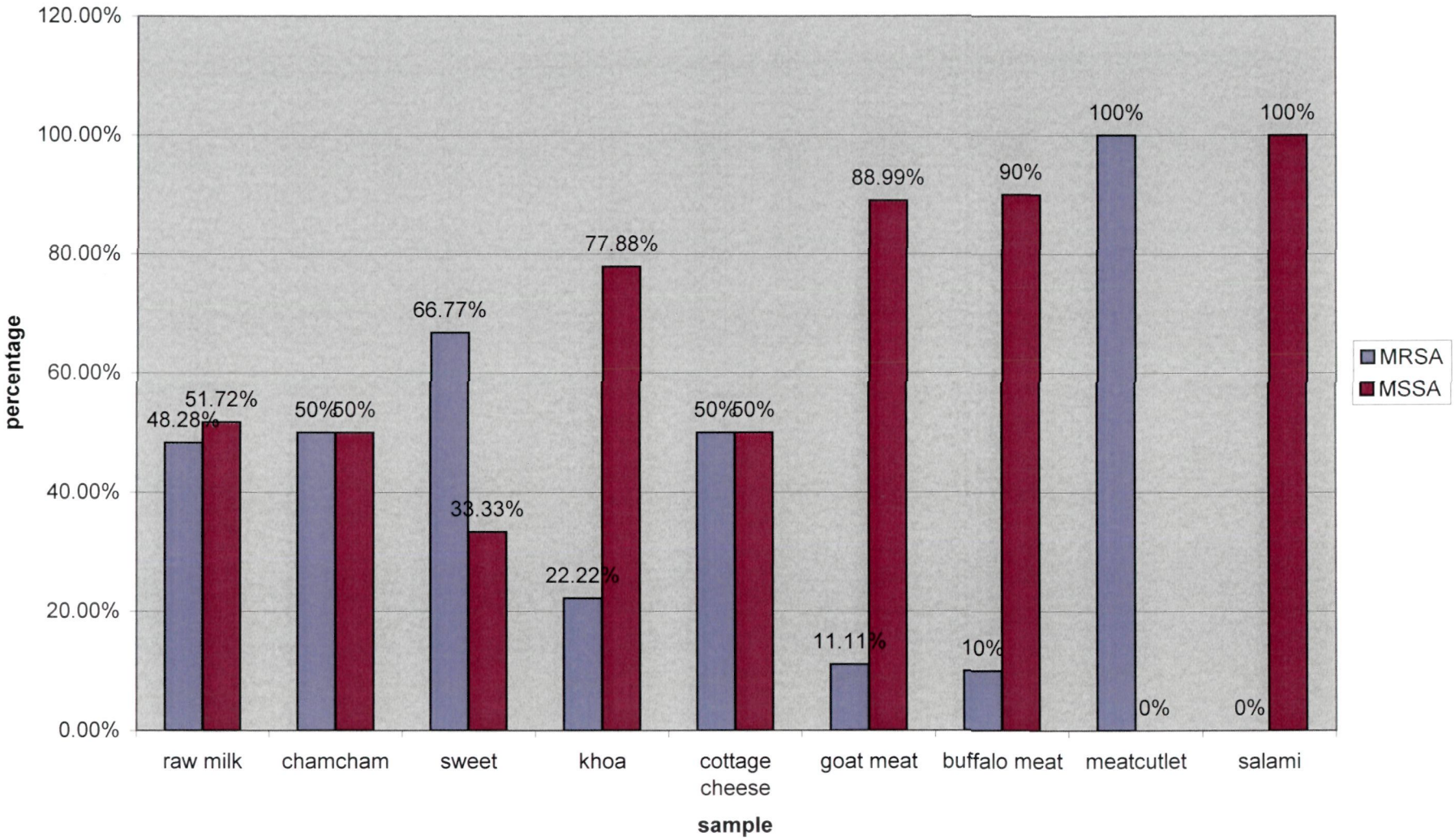
cottage cheese, buffalo meat and meat cutlet samples similar resistance pattern was found by all the three methods. However, in khoa only 2% isolates were observed methicillin resistant by PCR whereas other two methods failed to detect resistance. In goat meat only 2% isolates showed oxacillin resistance by disc diffusion and agar screen method but 3% were detected by PCR. The comparative detection of methicillin resistance by these three methods in relation to the food samples are shown in table – 20 (fig- 20).

Table- 20. Detection of Methicillin resistant *S. aureus* isolates from animal-origin isolates by various diagnostic methods

Samples	Total no. of isolates	Methicillin resistant isolates detected by		
		Disc Diffusion	Agar Screen	PCR
clinical specimens of animal origin (raw milk)	29	15 (15%)	14 (14%)	14 (14%)
Chamcham	4	2 (2%)	2 (2%)	2 (2%)
Sweet	3	2 (2%)	2 (2%)	2 (2%)
Khoa	9	0 (0%)	0 (0%)	2 (2%)
Cottage chesse	6	3 (3%)	3 (3%)	3 (3%)
Goat meat	27	2 (2%)	2 (2%)	3 (3%)
Buffalo meat	20	2 (2%)	2 (2%)	2 (2%)
Meat cutlet	1	1 (1%)	1 (1%)	1 (1%)
Salami	1	0 (0%)	0 (0%)	0 (0%)
Total	100	27(27%)	26(26%)	29(29%)

Figures in parentheses indicate percentage

Fig- 20 Bar diagram showing methicillin resistance in relation to various animal-origin food samples



DISCUSSION

5. DISCUSSION

Staphylococcus aureus is an important microorganism producing various diseases in man and animals. A variety of exoproteins produced by *Staphylococcus aureus* contribute to the pathogenesis in human and animal host (Salasia *et al.*, 2004). In man, it is an important cause for both nosocomial and community acquired infections like post-operative wound infections, pneumonia, bacteraemia, food poisoning and other infections. In animals it causes a wide spectrum of diseases like intramammary infections in lactating animals (Waage *et al.*, 1999; Cucarella *et al.*, 2004), sporadic septicemia in pigs, abscesses, sinusitis, arthritis, osteomyelitis, dermatitis. in poultry and ducks. The studies exploring the staphylococcal enterotoxin genes are elementary from our country and also the molecular studies characterizing the isolates at the genotypic level are fragmentary. Moreover, we could not get any study evaluating the relatedness or diversity of Staphylococcus isolates obtained from clinical and food samples. Therefore the present study was conducted specially to characterize the Indian *S. aureus* isolates at phenotypic and molecular level, to find out the existing enterotoxins, and to look for any relatedness or diversity of the isolates obtained from clinical and food sources.

Hemolysis is considered to be one of the important virulence markers of *Staphylococcus aureus*. The workers have reported β-hemolysis ranging from 86 to 97% (Kostrzynski and Kozanecki, 1990). In our study 77.4% human isolates showed β-hemolysis on sheep blood agar. The clinical isolates showing β-hemolysis also had multidrug resistance character.

59% of food isolates showed β-hemolysis on sheep blood agar in the present study. Kohler-Samouilidis (1977) reported 48% of *Staphylococcus aureus* of animal origin with hemolytic property on sheep blood agar. Rosec *et al.*, (1997) observed 30.5% hemolysing producing isolates among 231 food isolates of *Staphylococcus aureus.*

The ability of *Staphylococci* to produce free coagulase, an extra cellular enzyme which activates a coagulase reacting factor (CRF) is normally present in plasma and clots plasma by conversion of fibrinogen to fibrin, and contributes to its pathogenicity It has been proposed that coagulase may inhibit phagocytosis and protect the cocci from antibacterial substances in tissue fluids by laying down a fibrin barrier around them and rendering them resistant to opsonization and phagocytosis. Its production is the principal criterion used by the clinical microbiologists for the identification of *S. aureus* strains from human infections. Staphylocoagulase a major phenotypic determinants of *S. aureus* exists in multiple allelic forms, partly because of the existence of gene variants within the 3'-end coding region. This region contains a series of repeating 81-bp DNA sequences which differ both in the number of tandem repeats and the location of the *AluI* restriction sites among different isolates.

In our study, a comparative analysis of 202 isolates (102 human clinical isolates and 100 animal-origin isolates) was carried out to re-appraise the discriminatory power of the coagulase slide test, tube test and PCR. All of the human clinical isolates included in the present study, were found positive for coagulase enzyme by slide and tube coagulase test as well as by PCR. However, 98 isolates obtained from animal-origin samples were positive for tube coagulase test, slide coagulase test and

coagulase-PCR. Remaining four isolate were found positive by tube coagulase test and coagulase-PCR, but missed detection by slide coagulase test. Olney Vieira-da-motta *et al.*, (2001) reported 10.1% strains negative by the slide test. In another study, 3.9% of clinical and 10% of food isolates were found negative by slide test (Bachhil, 1998). The discrepancy could be because of detection targets, as slide coagulase test detect bound coagulase and tube coagulase test detect free coagulase.

In the present study, all the coagulase positive *Staphylococcus aureus* isolated from human clinical and animal-origin samples were further tested for the thermonuclease production on DNA-toluidine blue agar plates for confirmation of *Staphylococcus aureus.* It is an endonuclease, degrading both DNA and RNA, and the enzymatic activity can resist 100°C temperature for at least 1 hour. The TNase protein has been well characterized and its gene, the *nuc* gene has been cloned and sequenced. Thermonuclease production was observed in 100% of *S. aureus* isolates from human clinical specimens and animal-origin samples in this study. The results of present study were also supported by the findings of Jasper (1973), Lachica *et al.* (1971) and Das (1988), who reported that TNase is a reliable indicator for confirmation of *Staphylococcus aureus.*

In the present study **Nuc gene** was also detected by Polymerase chain reaction in all the human clinical and animal-origin isolates. Similar results were found by Kim *et al.* (2001). The *nuc* gene was found in all the strains obtained from animal sources especially, milk and milk products.

Accurate and rapid typing of *S. aureus* is crucial for control of infectious organisms and numerous methods have been described for

typing of *S. aureus* i.e. immunoblots, antimicrobial resistance and bacteriophage typing. Bacteriophage typing scheme for *S. aureus* was been introduced internationally from 1951. Although it is a cost–effective approach for typing of a large number of referred isolates the typeability is poor.

In this study, 70.58% human clinical isolates were found typeable by bacteriophage typing at 1 RTD and at 100 RTD. Among these, majority (35.3%) of them belonged to phage group III followed by 21.6% in phage group II and 10.9% in mixed group. Hanumanthappa *et al.*, (2003) found 61.43% typeable clinical isolates by using conventional phage typing and observed that majority of isolates 55.81% were typed in phage group III. While in another Indian study by Kandle *et al.*, (2003), out of 289 clinical isolates 113 (39.11%) strains could be typed. Among the typeable strains 5.53% belonged to phage group I, 11.4% to group II, 8.9% to non allocated group and majority of 13.14% strains were typed in group III.

Majority of MRSA were non-typeable by conventional sets of phages and hence these isolates were typed using a new set of MRSA phages. In the clinical isolates typeability of 41.18% at 1 RTD and 100 RTD was seen among 34 methicillin-resistant *Staphylococcus aureus*. Briefly, at 1 RTD out of 15 pus isolates, 3 (20.0%) were typeable and belonged to mixed group; 4 (100%) from Cx. swab were found typeable (one in group III and 3 in mixed group); and 1 (100%) from drain tips (group I). Whereas all isolates from urine, conj. swabs, ear swabs, throat swabs, stool, vomitus, Body fluid and CSF were found nontypeable at 1 RTD.

However at 100 RTD two pus isolates, out of 12, were typeable in mixed group; 3 urine isolates (one belonged to group II and the other to group III), one vomitus (group III) were found typeable, whereas rest could not be typed. Similarly, Vidhani *et al.* (2003), studied 188 *S. aureus* isolates from wound infection of which 79 were MRSA. Using MRSA phages at 100 RTD 41.8% of MRSA isolates could be typed.

Mathur *et al.* (2000), studied 300 MRSA clinical isolates, of which 17.6% could be typed by conventional set of phages and group III predominated. When tested with MRSA phages 45.6% strains could be typed into 5 phage patterns. Phage 622 was predominant followed by M3/M5. MR8/MRP MR 25, 30/33/38 and mixed group.

In animal-origin isolates, 2% isolates were found typeable using conventional phages at 1 RTD and 100 RTD belong to group II. 195 *S. aureus* isolates were studied in Canadian dairy herds by Sabour *et al.* (2004). 63.1% of them were lysed by phage group I and III while 32.8% could not be typed.

S. aureus has been recognized as an important pathogen in human and animal diseases. Emergence of antibiotic resistance is a major problem all over the world. Constant and irrational use of antibiotics in both human and veterinary medicine, especially in developing countries coupled with current knowledge of transfer of antibiotic resistance between various bacteria makes it essential to monitor the susceptibilities of known pathogenic bacteria to antibiotics (Linton, 1997). Increasing trend in irrational usage of antibiotics as therapeutic and growth promoter in veterinary practice also leads to multidrug resistant *S. aureus.* Similarly indiscriminate usage of antibiotics and non-compliance to the antibiotic treatment in human beings are some of the important causes of

increasing drug resistance observed among various microbes (Linton, 1977).

Although antimicrobials were initially very effective, but the fast emergence of drug resistant *Staphylococcus aureus* strains have created a problem in the control and treatment of various infections.

In the present study, 98.03% of *S. aureus* isolates from **human clinical specimens** showed resistance to penicillin. Most of the reports from previous studies have revealed that *S. aureus* from various sources had resistance to penicillin ranging between 59.89% to 98.96% (Das, 1988; Sanjeev and Mahadeva Iyer, 1988; Kar *et al.* 2003; Norazah *et al.* 2003; Shamsuzzarman *et al.* 2003 and Audu and Kudi, 2004). In an study, Murugan *et al.*, (2008) noted 100% penicillin resistant strains among 2314 clinical isolates of *S.aureus*.

Resistance to chloramphenicol was observed in 23.53% strains in the present study. This is comparable to 23.1% resistance obtained by Archer *et al.* (1991), Orrett (2008) noted 13.3% resistance but Refsahl *et al.* (1992), reported higher resistance (79.4%) to chloramphenicol. In our study 64.7% strains were resistant to amoxycillin. Assadullah *et al.* (2003), and Vidhani *et al.* (2001) reported 89.3% and 100% resistance for amoxycillin. Among cephalosporins, in the present study, 32.35% strains were resistant to cefaclor, cefotaxime, ceftazidime and cefepime.

In this study, 32.35% were found resistant to oxacillin. Vidhani *et al.* (2001) found 57.6% methicillin resistant *Staphylococcus aureus,* Anupurba *et al.* (2003) reported 54.8% MRSA, Majumder *et al.* (2001) reported 52.9% MRSA from patients and healthy carriers. Whereas in a study done by, Murugan *et al.*, (2008), noted 42.86% MRSA among patients. Cotrimoxazole resistance was found to

be 69.61% in present study, whereas Pulimood *et al.* (1996) and Assadullah *et al.* (2003) and Orrett 2003) reported cotrimoxazole resistance in 97.1%, 53.5%and 93.6% strains respectively. Pal *et al.* (1991), reported cotrimoxazole resistance in 89.6% strains.

Amongst aminoglycosides in the present study, 27.45% and 35.3% strains were resistant to gentamicin and amikacin respectively. Mathur *et al.* (2000), reported 44.5% and 38% and Orrett (2008)noted 96.8% and 94.1% resistance to gentamicin and amikacin respectively. In other studies, variable resistance to gentamicin has been reported (Assadullah *et al.* 2003; and Anupurba *et al.* 2003).

Among the fluoroquinolones, 60.79% strains were found resistant to ciprofloxacin. This is in comparison to the study of Mathur *et al.* (2000), Assadullah *et al.* (2003), Anupurba *et al.* (2003) and Orrett (2008) who reported resistance in 50.8%, 45.8%, 84.1% and 95.2% isolates respectively.

Resistance to erythromycin was observed in 54.9% of strains. This was in accordance with the findings of Majumder *et al.* (2001), who reported resistance to erythromycin in 33.9%. Mathur *et al.* (2000), reported resistance to erythromycin in 92% isolates respectively. Orrett (2008) noted 98% resistance erythromycin strains.

In the present study, out of 16 antibiotics tested, a multidrug resistance phenomenon was observed among the **animal-origin isolates.**

The frequency of resistance to different antibiotics were penicillin (93%), tetracycline (49%), erythromycin (51%), ciprofloxacin (39%), chloramphenicol (34%), amikacin (27%), cefaclor (27%), ceftriaxone (27%), ceftazidime (27%), cefepime (27%), oxacillin (27%), gentamicin

(20%), cotrimoxazole (54%) and amoxycillin (19%). These isolates were found sensitive to vancomycin and teicoplanin.

In this study, 93% of *S. aureus* isolates from **animal-origin sources** showed resistance to penicillin Previous studies have shown varying resistance to penicillin ranging from 59.89% to 98.96% (Das, 1988; Kar *et al.,* 2003; Norazah *et al.,* 2003; Audu and Kudi, 2004 and Chao *et al.*, 2007).

Overall, tetracycline resistance was 49% in this study with varying resistance (0-70%) among isolates obtained from food sources like raw milk, raw meat, cooked meat and food products made by milk. Similar resistance profile (63.0% to 90.8%) has been reported by many other workers (Gonlugur *et al.* 2003; Norazah *et al.* 2003; and Audu and Kudi, 2004). Chao *et al.*, (2007) noted 49.4% resistance among animal specimens The resistance to cotrimoxazole, ciprofloxacin and chloramphenicol in this study was found in 54%, 39% and 34% isolates respectively.

In this study the resistance to amikacin, gentamicin and amoxicillin was noticed in, 27%, 20% and 19% strains respectively. Martin *et al.* (2003), reported 38.1% resistance to amoxycillin in food isolates. For gentamicin, varying resistance (2.5 to 98.3%) had been reported by various workers (Corti *et al.,* 2003; Norazah *et al.,* 2003; and Shamsuzzaman *et al.,* 2003).

In the present study, 27% of *S. aureus* isolates from food samples showed resistance to cefaclor, cefotaxime, ceftazidime and cefepime. Methicillin resistance was obtained in 27% isolates. A varying prevalence rate of MRSA (6.9% to 52.9%) has been reported (Chakravarthy *et al.* 1988; Udayasankar *et al.* 1997; Mehndiratta *et al.,* 2001; Vidhani *et al.*

2001; and Hanumanthappa *et al.,* 2003; Saxena *et al.,*2003; Anupurba *et al.,*2003; and Kim *et al.,* 2004 and Tiwari *et al.,* 2008). The varying prevalence rates may be due to variation in the nature of isolates tested, methods of detection used in different geographical areas

Methicillin resistant *Staphylococcus aureus* (MRSA) strains were identified as early as 1961 immediately after the introduction of methicillin in clinical settings (Barber, 1961). These strains have probably arisen by a succession of mutations and the acquisition of resistance plasmids. MRSA strains have emerged worldwide as a major cause of nosocomial infections (Michel *et al.* 1997). Methicillin resistant Staphylococcus aureus is also an emerging issue in veterinary medicine (Defra, 2008). MRSA strains are difficult to eradicate because of their multiple drug resistance, thereby complicating the management of infections in animals too. The need to differentiate MRSA from MSSA in clinical specimens. This demands a rapid and accurate method of MRSA detectionfor proper management and prevention of transmission of *S. aureus* in clinical settings. Though various methods for detection of MRSA have been recommended, the heterogeneous nature of resistance to methicillin and oxacillin by many strains make their recognition problematic. In this study for detection of Methicillin resistance various conventional methods (disc diffusion and screen agar plate) and polymerase chain reaction were used. Among the 102 **human clinical isolates,** percentage of MRSA detected by disc diffusion method, screen plate agar method and PCR was very similar (31 to 33.4%).

Adaleti *et al.* (2008), studied 416 clinical isolates, 210 (51%) isolates were found positive for *mec* A gene by PCR. In their study, 26 *mec* A negative strains were falsely identified as MRSA by disc diffusion

method and by Screen agar plate method. However, in our study we missed only one *mec* A positive isolate as MRSA by disc diffusion and 2 isolates by screen agar method. These variations might be because of variations in the phenotypic expression of oxacillin resistance gene such as heterogenous expression of methicillin-resistance.

Among the 100 **animal-origin isolates,** percentage of MRSA detected by disc diffusion method, screen plate agar method and PCR was very similar (26% to 29%).The results of this study of MRSA by PCR seem to be promising for early and reliable identification of methicillin-resistant *Staphylococcus aureus.*

For rapid detection of MRSA targeting of *mec* A gene by PCR has been used by several workers (Suzuki *et al.* 1992; Jonas *et al.* 2002; and Louie *et al.* 2002). The reported *mec* A gene positivity in *S. aureus* was between 33.07% to 66.66% (Vannuffel *et al.* 1995 Louie *et al.* 2000; Louie *et al.* 2002; Merlino *et al.* 2002 and Haveri *et al.*, 2008).

In the present study sensitivity for vancomycin and teicoplanin was found in 100% human clinical and animal-origin isolates. This finding is in accordance to Kim *et al.* (2003), Assadullah *et al.* (2003) and Murugan *et al.*, (2008). Nosocomial infections with *Staphylococcus aureus* necessitate prompt recognition of the infectious chain as well as a rapid investigation and exclusion of infectious sources.

The conventional typing procedures (e.g. phage typing) and genotyping method like pulsed–field gel electrophoresis (PFGE) are labor intensive, time consuming and can be performed only in few laboratories. An attractive typing technique for *S. aureus* utilizes the polymorphism of the coagulase (*coa)* gene as an epidemiological marker. This is simple, rapid, discriminatory and reproducible method for typing this pathogen

(John, 2002). The range of patterns with in a single phage type of *S. aureus* could help to discriminate between isolates. In a hierarchical approach *coa* gene RFLP could occupy an intermediate position between phage typing and PFGE. This study demonstrates the value of PCR-RFLP (*AluI*) analysis of the coagulase gene for the rapid initial genotyping of *S. aureus* isolates, particularly of human and animal origin.

Twelve RFLP patterns of clinical isolates of *S. aureus* were obtained in the present study. Majority 30 (29.4%) of strains showed bands of 243 bp and 405 bp were classified in group XII followed by 22 strains (21.6%) in group III showing bands of 81 bp, 243 bp while 10 strains (9.8%) were characterized by a distinctive bands pattern of 162 bp and 243 bp and were characterized in group VI. Eight strains (7.8%) having bands of 405 bp and 567 bp were classified in group IX. Seven strains (6.9%) each showed bands of 81bp, 162 bp, 243 bp, 324 bp, 405 bp and 162 bp, 324 bp, 405 bp, respectively, and were categorized in group I and group VIII. Five strains (4.9%) and 4 strains (3.9%) each showed bands of 81 bp, 162 bp: and 162 bp and 324 bp respectively, were classified in group VII and group II, 3 strains (2.9%) each were classified in group IV and group X consisting of band patterns of 162 bp, 243 bp, 324 bp, 567 bp and 162 bp, 332 bp, 405 bp, respectively. Two strains (1.9%) showed band patterns of 162 bp, 243 bp. 405 bp belonged to group XI. However, 1 strain (1.0%) of group V showed band pattern of 243 bp and 324 bp.

Eighteen out of 38 pus isolates (47.37%) were classified in group VI with band patterns of 81 bp, 162 bp and 243 bp.

Variability in *coa*-RFLP patterns have been reported in the literature. Shittu *et al.*, (2006), observed 11 Coa-RFLP patterns among

227 clinical specimens. Goh *et al.* (1992), observed 10 distinct RFLP patterns out of 30 *S. aureus* strains. He also observed additional *S. aureus* strains from local hospital (24 MRSA and 15 MSSA) out of which 25 MRSA, showed the same pattern while 1 was of a different pattern and 15 MSSA showed 8 pattern and 2 were not amplified.

Schwarzkopf *et al.* (1994), got 10 different RFLP patterns from 150 strains of *S. aureus* isolated from clinical specimens. Hookey *et al.* (1998), observed 95 strains of *S. aureus* and found 10 distinct RFLP patterns. Shopsin *et al.* (2000), observed 13 unique *coa* patterns whereas Chiou *et al.*(2000), studied 71 *S. aureus* isolates recovered from nine food- borne outbreaks and found 11 *coa*-RFLP patterns. Montesions *et al.* (2002), observed PCR–RFLP pattern of 124 MRSA isolated from university hospital of he Canay Island and found five patterns.Wang *et al.* (2003), observed 229 *S. aureus* from 36 food borne outbreaks at Taiwan and confirmed them as *S. aureus* by tube coagulase test. 10 SEC producing strains were screened for the r pattern by PCR–RFLP using enzyme and found 4 types of pattern. Scherrer *et al.* (2004), observed 191 isolates of *S. aureus* and found different patterns by using AluI.

Among these 12 patterns observed, 7 group patterns, namely, group I, group II, group IV, group V, group VI, group VIII and group XI were noticed in MRSA isolates. Rest of the group patterns were found associated with MSSA isolates. Briefly, out of 34 *Staphylococcus aureus* isolates from clinical specimens 7 (20.6%) belonged to *Coa*-RFLP pattern of group I where majority of strains were resistant to six or more antibiotics; 10 strains (29.4%) belonged to *Coa*-RFLP group VI and 4 strains (11.8%) of *Coa*-RFLP group I. were found resistant to six or more antibiotics. While 7 (20.6%) strains belonged to *Coa*- RFLP group

VIII having resistance to five or more antibiotics. *Coa*-RFLP group IV and group XI showed resistance to five or more antibiotics and comprised of 3 strains (8.8%) and 2 strains (5.9%) respectively, however, only one strain (2.9%) showed resistance to five antibiotics which belonged to *coa*-RFLP group V.

Among these 12 patterns, 5 groups patterns, namely, group III, group VII, group IX and group X group XII were noticed in MSSA isolates. Antibiotic resistance patterns in relation to Coa – RFLP pattern could not be inferred in isolates obtained from clinical specimens due to variability of resistance patterns; 40 different types of patterns were noticed in these isolates. Majority 10 (9.8%) of the MRSA isolates showed Coa- RFLP pattern of group VI, followed by group I (6.9%) and group II (3.9%). However, no predominant resistance patterns were noticed.

In 100 animal-origin isolates 7 types of *coa* -RFLP were observed. A majority of strains (47%) were classified in group VI with bands of 405 bp followed by 17% strains characterized by distinctive band patterns of 243 bp and 324 bp in group V, 13% strains in group I with a band pattern of 162 bp, 250 bp and 405 bp , 8% strains showed bands of 81 bp and 324 bp and belonged to group VII. and 7% strains showed bands of 324 bp and were characterized as group II, 3% strain showed a band pattern of 162 bp and 405 bp classified in group IV. One percent strain of group III showed band patterns of 162 bp and 324 bp. Five each out of 29 raw milk isolates were classified in group VII and group II with band patterns of 81 bp, 324 bp and 324 bp. To appreciate the reproducibility of coa-RFLP the test were performed in duplicate. The reproducibility was good as similar result were obtained in both the tests.

In the present study, *S. aureus* isolates from animal-origin samples and human clinical specimens were found to differ in their gene patterns whereas animal clinical specimens and animal-origin food samples showed mixed Coa-RFLP gene patterns. This finding might provide a better understanding of the distribution of the *S. aureus* clones among human and animal-origin isolates and can be helpful in the control of *S. aureus* infections.

Marcos *et al.* (1999), studied *S. aureus* isolates from human, cows and sheep and got two different RFLP patterns. Motta *et al.* (2001), observed 555 milk samples and compared coa gene by phenotypic and genotypic method. He found 50% positive coagulase staphylococci by phenotypic methods and 128 *S. aureus* classes according to size of PCR product. Cucarella *et al.* (2004), observed 195 *S. aureus* from sub–clinical case of mastitis among cows of Valencia and were identified as *S. aureus* by coagulase tests. Molecular typing was done using PCR–RFLP and 10 distinct patterns were observed. Salasia *et al.* (2004), studied 35 food isolates and they showed different *Coa*-RFLP patterns.

Holeckova *et al.* (2004), studied 110 samples of raw sheep milk and reported 46 (41.8%) coagulase positive (by phenotypic method) out of which 6 (13%) were found to be enterotoxigenic. Kalorey *et al.* (2007), studied 37 food isolates and they showed three different *Coa*-RFLP patterns.

Among these 7 Coa-RFLP patterns, 4 group patterns, namely, group II, group III, group I, and group VII, were noticed in 29 MRSA isolates from food samples. Rest of the group patterns were in MSSA isolates. Briefly, out of 29 *Staphylococcus aureus* isolates from animal-origin samples 8 (27.6%) belonged to *Coa* - RFLP pattern of group VII

where majority of strains were resistant to six antibiotics. 13 (44.8%) strains belonged to *Coa* - RFLP group I with resistance to five antibiotics. *Coa*-RFLP group III exhibiting resistance to seven or more antibiotics was in 7 strains (24.1%). Only one strain (3.4%) showed resistance to ten or more antibiotics which belonged to *Coa*-RFLP group II. Among these 7 patterns, 3 group patterns, namely, group IV. group V, and group VI were noticed in MSSA isolates. Antibiotic resistance patterns in relation to *Coa* – RFLP pattern could not be inferred in isolates obtained from food samples due to variability of resistance patterns; 40 different types of patterns were observed. Majority 13 (44.8%) of the MRSA isolates showed *Coa*- RFLP pattern of group I followed by group VII (27.6%) and group II (2.4%). However, no predominant resistance patterns were noticed.

Results of coagulase gene typing demonstrated that the MRSA and MSSA strains from clinical specimens were classified into 7 and 5 *Coa*-RFLP patterns, respectively. However, in animal-origin isolates 4 MRSA and 3 MSSA *Coa* - RFLP patterns were observed. The ability of the PCR-RFLP typing method of the coagulase gene to differentiate between MRSA and MSSA in human clinical and animal-origin isolates was also noted. The MRSA and MSSA strains did not share similar PCR-RFLP patterns. MRSA gave distinct *Coa*-RFLP patterns as compared to those of MSSA. We feel that *Coa*-RFLP technique could be incorporated as diagnostic tool to confirm the MRSA. It's also observed that the *Coa*-RFLP patterns of the MRSA strains were unique and distinct from the MSSA strains from both types of the isolates of *Staphylococcus aureus*. *Coa*-RFLP is performed with primers, homologous to a conserved region with in the *coa* gene, in order to amplify the sequence

encoding the C-terminals region of this molecule. Since the number of repetitive sequence varies within the *coa* gene, the resulting PCR products of individual strains can be of different lengths. Therefore we found different length and different patterns of *S. aureus* strains in *coa* RFLP. It is noted that there is extensive polymorphism with *coa* gene circulating in human and animal strains. We believe that the heterogeneity observed for the *coa* gene has a potential discriminatory power for further epidemiological studies of medical and veterinary importance. Further molecular studies are required particularly at the sequence level to study whether the reported PCR product size classes represent distinct *S. aureus* strains. Shitu and Lin, (2006), also found coa-RFLP of the coagulase gene able to distinguish MSSA from MRSA and offers an attractive option to be considered in the rapid epidemiological analysis in South Africa.

There is paucity of Indian literature regarding the Coa-RFLP especially on *Staphylococcus aureus* of clinical and animal origin food isolates. This is a premium study of this kind. Because of its ease, speed and good reproducibility it can be considered for epidemiological typing.

It was perceived that Coa-RFLP can be used as an adjunct to bacteriophage typing. The strains which were untypeable by bacteriophage typing were easily typed by Coa-RFLP. Thus in a resource poor country like India, the first line typing method for clinical isolates may still be bacteriophage typing which can be supplemented by Coa-RFLP . in this study Coa-RFLP was extremely useful in typing the food isolates which were virtually untypeable by bacteriophage typing. Further Coa-RFLP showed good discrimination between clinical and food isolates. In this study MRSA and MSSA typeability was again far better

with Coa-RFLP. Coa-RFLP can also be a useful tool for continuous surveillance of resistance patterns and characterization.

The differences in Coa-RFLP patterns in the studies mentioned point to distinct region to region variation in the *Staphylococcus aureus* strains. This difference can be utilized for the purpose of genotyping as mentioned in some studies (Shittu *et al.*, 2006).

S. aureus is considered to be an important cause of food poisoning because of production of staphylococcal enterotoxins (SEs). So far at least, 20 serologically distinct SEs have been identified. SEA, SEB, SEC, SED, SEE (Jones and Khan, 1986; Betley and Mekalanos, 1988; Couch *et al.*, 1988; and Bayles and Landolo, 1989) represent classical types, while SEG, SHE, SEI and SEJ are newly described enterotoxins (Ren *et al.*, 1994; Su and Wong, 1995; Munson *et al.*, 1998; and Zhang *et al.*, 1998). SEC has been described with minor antigenic variations and designated SEC1, SEC2 and SEC3 (Bergdoll *et al.*, 1965; Avena and Bergdoll 1967; and Reiser *et al.*, 1984). Recent studies (Jarraud *et al.*, 2001; Orwin *et al.*, 2001; Letterer *et al.*, 2003; and Omoe *et al.*, 2003) have described other SE genes (*se*); *sek, sel, sem, sen, seo, sep, seq, ser* and *seu* which point to the possible existence of new staphylococcal enterotoxin (SE). Not all staphylococci are SE producers, or the amount of produced SE may be insufficient for food intoxication. Among all the enterotoxins, SEA, SEB and SEC are reported to be the most common types implicated in food poisoning episodes (Asao *et al.*, 2003; Chen *et al.*, 2001; and Tkacikoca *et al.*, 2003). Rasooly *et al.*, (1999) reported SEA as most commonly involved enterotoxin in out breaks of *staphylococcal* food poisoning in man. According to Wilson (1991), and Mc I auchlin *et al.*, (2000), SEC is

commonly associated with bovine, ovine and caprine dairy products. It is also reported that SEC was the most frequently found staphylococcal enterotoxin produced by animal strains (Hajek, 1978) in comparison with human strains. In this study, the samples were tested for commonly encountered enterotoxin A, B and C. In the present study, out of the 102 human clinical isolates 3 (2.94%) isolates were found enterotoxogenic, out of which 1 from the stool sample was found positive for enterotoxin A, 1 vomitus sample showed presence of enterotoxin B while 1 from urine showed presence for enterotoxin C. Flemming *et al.* (2007), studied 2,72' stool samples out of which 36 SEA; 20 SEB; 19 SEC; 68 SED and 2 SEE were found. In a study from Central Taiwan Chen *et al.* (2001) found SEC as a major subtype for *S. aureus* strains isolated from fecal samples from randomly selected diarrhoeal patients associated with food borne out - breaks during 1995 - 2000. Whereas, Klotz *et al.* (2003), studied 93 clinical isolates from stool samples and found 58.1% enterotoxogenic strains (SEA in 12.9%, SEB in 9.7%, SEC in 21.5% and SED in 14%). Adwan *et al.* (2008), studied 33 urine and 24 semen samples and found 27.3% and 12.5% enterotoxogenic strains, respectively, out of theses samples SEC gene found along or in combination with other genes.

Among the 100 **animal-origin isolates**, a total of 13 were enterotoxigenic. Of these 2% were positive for enterotoxin A (1 from raw milk sample and 1 from buffalo meat); 3% were positive for enterotoxin B (1 from raw milk sample, 1 from sweet and 1 from cottage cheese) and 8% were found positive for enterotoxin C (1 from raw milk, 1 from paneer, 3 from goat meat and 3 from buffalo meat). Holeckova *et al.* (2004), tested 46 raw sheep milk samples; of which 13% were found as

enterotoxigenic (8.7% were SEA, 2.2% SEB, and 2.2% SEA+ SEB producers). Tkacikova *et al.* (2003) reported 36.8% enterotoxogenic isolates from 87 food samples, out of which 17.2% were positive for SEC and SED, 1.1% SEA and SEB each. In another study by Scherrer *et al.* (2004), they found 65.2% enterotoxigenic *S. aureus* out of 293 isolates from bulk-tank milk samples of goat and sheep. The occurrence of SEC, SEG, SEJ, SEI, SEB and SED was noticed in 42%, 10.6%, 8.9%, 8.2%, 1.4% and 1.4% respectively. Rosec *et al.* (1997) conducted a study on 213 *S. aureus* isolates from clinical and food samples and reported 30.5% as enterotoxigenic isolates. SEC was reported as predominant type in the strains belonging to human biovar. Valle *et. al.* (1990) reported 74.3% enterotoxigenic isolates from different anatomical sites in healthy goats. SEC was the most frequently produced enterotoxin either alone (67.9%) or in combination with others i.e. SEA + SEC 2.7%, SEB+SEC 2.7% SEC +SEE 2.7%, SEA +SEB +SEC 0.9%. Loncarevic *et al.* (2005), studied 386 *S. aureus* isolates from raw milk and their products and found 48% and 51% enterotoxogenic strains, in milk and milk products respectively.With respect to various raw or cooked food stuffs, enterotoxigenicity amongst *S. aureus* strains ranged from 16 to 86% (De Buyser *et al.,* 1985; and Ewald, 1987). Holeckova *et al.* (2002), showed variable results regarding the occurrence of enterotoxigenic *S. aureus* strains in food stuffs probably due to the difference among the kinds of food items examined, number of samples tested, detection methods used and in ecological origin of strains. Nema *et al.*, 2007 found a combination of SEB and SED combination enterotoxins among food samples.

Among the 16 enterotoxigenic *Staphylococcus aureus* isolates, 13 different antibiotic resistance patterns were observed in this study. All of the enterotoxigenic isolates from human clinical specimens were found resistant to methicillin. However both types of strains exhibited different antibiotic resistance patterns. Out of 13% enterotoxigenic animal-origin isolates 10 different antibiotic resistance patterns were observed. 8% of them were noted as MRSA out of which presence of enterotoxin A was noticed in 2% isolates, SEB in 2% and SEC in 5% isolates. Similarly, Schmitz *et al.* (1998) reported 24% enterotoxigenic MRSA strains and found SEB in 7% and SEC in 11%.

Since there is paucity of Indian literature on prevalent enterotoxins, especially in human clinical isolates, the present study would be able to enlighten the Indian scenario.

SUMMARY & CONCLUSIONS

6. SUMMARY AND CONCLUSION

Staphylococcus aureus is an adaptable, opportunistic pathogen, its ability to persist and multiply in a variety of environments leads to wide spectrum of diseases in both humans and animals. In humans *Staphylococcus aureus* is the causative agent of many infections, ranging from superficial skin suppurations to life-threatening septicaemias associated with visceral or bone infections. Successful treatment is often hindered by the increasing prevalence of methicillin-resistant strains and by antibiotic inefficacy against the bacteria involved in chronic infections.

In lactating female animals, *Staphylococcus aureus* is a common cause of intramammary infections, frequently leading to chronic mastitis. Because this type of infection is very difficult to eradicate with antibiotic therapy, a premature culling of animals, involving substantial production losses, is the only efficient strategy to control this type of mastitis.

Various global studies are available characterizing the *Staphylococcus aureus* at molecular level, however, the studies are fragmentary from India on the current aspect and especially in *Staphylococcus aureus* isolates obtained from samples of animal origin.

Therefore, the present study was undertaken with the aim to evaluate the phenotypic and genotypic characters of *Staphylococcus aureus*, which might help to understand the characters of Indian *S. aureus* isolates obtained from human infections and from food samples of animal origin.

A total of Three thousand five hundred and fourteen clinical specimens and 1219 animal origin food samples were collected during June 2003 to March 2006. Of these 102 *Staphylococcus aureus* from

human clinical and 100 from animal origin samples were randomly selected for further study. Out of 102 *Staphylococcus aureus* studied from **clinical specimens**, 38 were from pus samples, 10 from urine samples, 1 from CSF, 2 from I/V catheter tips, 3 from body fluids, 3 from ear swabs, 3 from throat swabs, 14 from conjuctival swabs, 14 from cervical swabs, 7 from stool samples, 6 from vomitus samples and 1 from semen specimen. Out of 100 **animal-origin samples**, 29 *Staphylococcus aureus* were obtained raw milk, 4 from chamcha·n (a sweet prepared from milk), 3 from other sweets, 9 from khoa (milk concentrate used in sweets), 6 from paneer (cottage cheese), 27 from raw goat meat, 20 from raw buffalo meat, 1 from kabab (Meat cutlet) and 1 from salami sample were included in this study.

98.03% Clinical and 98% animal-origin isolates were found positive for slide coagulase test whereas they showed 100% positivity by tube coagulase test and by amplification of coagulase gene.

Out of 102 **clinical isolates** 80 (78.4%) were β-lactamase producers whereas of 100 **animal-origin isolates** 69 (69%) were found β-lactamase producers by iodometric method.

Among 102 **clinical isolates** maximum number of isolates were resistant to penicillin 100 (98.03%) followed by cotrimoxazole cotrimoxazole 71 (69.61%), tetracycline 70 (68.63%), amoxycillin 66 (64.7%), ciprofloxacin 62 (60.79%), erythromycin 56 (54.9%), amikacin 35 (35.3%), oxacillin 33 (32.35%), cefaclor 33 (32.35%), ceftriaxone 33 (32.35%), ceftazidime 33 (32.35%), cefepime 33 (32.35%), chloramphenicol 24 (23.53%) and gentamicin 28 (27.45%). While none of the isolates were found resistant to vancomycin and teicoplanin.

The isolates from **animal-origin samples** showed resistance to penicillin 93 (93%), erythromycin 51 (51%), tetracycline 49 (49%),

ciprofloxacin 39 (39%), cotrimoxazole 54 (54%), chloramphenicol 34 (34%), amikacin 27 (27%), oxacillin 27 (27%), cefaclor 27 (27%), ceftriaxone 27 (27%), ceftazidime 27 (27%), cefepime 27 (27%), gentamicin 20 (20%), and amoxycillin 19 (19%) in descending order. Only one isolate was found susceptible to all 16 antibiotics and none of the strains were found resistant to vancomycin and teicoplanin.

The drug resistance patterns of **clinical isolates** showed resistance to two drugs in 3 (2.94%) isolates, three drugs in 10 (9.8%) isolates, four drugs in 18 (17.65%) isolates, five or six drugs in 25 (24.51%) isolates, seven or eight drugs in 16 (15.67 %) isolates whereas resistance to more than ten or more drugs was found in 30 (29.47%) isolates. No isolate was resistant to only one drug.

In **animal-origin samples** the drug resistance patterns showed resistance to two drugs in 9 (9.0%) isolates, three drugs in 18 (18.0%) isolates, four drugs in 16 (16.0%) isolates, five or more than five drugs in 25 (25.0%) isolates whereas resistance to more than ten or more drugs was found in 23 (23.0%) isolates. One isolate was found sensitive to all 16 drugs.

Antimicrobial resistance has been noticed as one of the paramount microbial threats of the twenty-first century. *Staphylococcus aureus* has always been a stumbling block for antimicrobial chemotherapy and the introduction of new classes of antimicrobial agents is usually followed by the emergence of resistant forms of *Staphylococcus aureus*. Therefore, continuous surveillance on the resistance patterns and characterization of *S. aureus* in understanding new and emerging trends in human and animal from India is of utmost importance.

Methicillin resistant *Staphylococcus aureus* (MRSA) is a major nosocomial pathogen worldwide and is often difficult to detect due to the heterogeneous nature of expression of oxacillin resistance. A total of 102 clinical and 100 animal origin food isolates were tested for oxacillin resistant by disc diffusion method, by screen agar plate and detection of *mec* A gene by PCR. For the detection of *mec* A gene by PCR, the primers described by Prasad *et al.* (2000) were used, which gave a clear cut specific 604 bp amplified product.

Out of 102 **clinical isolates** 33 strains were oxacillin resistant by disc diffusion method, 32 strains by screen agar plate method and 34 strains were found positive for *mec* A gene by PCR.

In this study, 29% isolates from **animal-origin samples** were identified as MRSA by PCR, 27% by disc diffusion method and 26% by oxacillin agar plate method. The results of our study on PCR of MRSA seem to be promising for early and reliable identification of MRSA.

Bacteriophage typing was performed in 102 **clinical isolates**. Out of these isolates tested, only 55 (53.9%) isolates could be typed by the conventional set of phages at RTD. Distribution of isolates into phage groups revealed that maximum number of isolates 25 (24.51%) were typed in group III followed by 20 (19.61%) in group II, 7 (6.9%) in mixed group and (2.94%) isolates in group I. None of the isolates was typed at non allocated group.

47 non typeable isolates were further tested at 100 RTD, they showed 16.67% more typeability. Maximum number of these isolates belonged to group III (23.4%) followed by 8 5% in mixed group and 4.3% in group II.

The total typeability at 1 RTD and 100 RTD was observed in 70.58% of clinical isolates including 10 methicillin-producing strains.

MRSA phage groups were used to type methicillin resistant *Staphylococcus aureus* isolates of clinical specimens. A total of 14 (41.18%), out of 34 MRSA, were found typeable at RTD and 100 RTD using MRSA phages. Briefly, at RTD out of 15 pus isolates, 3 (20.0%) were found typeable and belonged to mixed group; 4 (100%) from cx. swab were found typeable (one in group III and 3 in mixed group); and 1 (100%) from drain tips (group I). Whereas all isolates from urine, conj. swab, ear swab, throat swab, stool, vomitus, Body fluid and CSF were found non typeable at RTD. The non typeable isolates at RTD could be typed at 100x RTD and on addition 16.6% isolates were typed. Briefly, at 100x RTD two pus isolates, out of 12, were typeable at mixed group; 3 urine isolates (one at group II and two at group III), one vomitus (at group III) were found typeable, whereas rest were non typeable.

Out of 100 **animal-origin isolates** none of the isolates were typeable at routine test dilution and at 100 RTD. MRSA phage groups were used to type methicillin-resistant *Staphylococcus aureus* isolates of food samples. Out of which only two isolates (one from milk and the other from buffalo meat) could be typed at 100 RTD which belonged to phage group II.

Two hundred and two coagulase positive *Staphylococcus aureus* isolates including 102 clinical and 100 animal-origin isolates were tested for the presence of thermostable nuclease (*nuc)* gene by polymerase chain reaction using Brakstad *et al.* (1992) method. All the 202 isolates showed a 270 bp amplified product after gel

electrophoresis, which is specific for the presence of *nuc* gene in *Staphylococcus aureus.*

The detection of the ***coa*** gene was done by Polymerase Chain Reaction using the primer set described by Goh *et al.* (1992). Twelve different electrophoretic patterns were observed in these isolates. Thirty strains (29.4%) showed a distinctive bands of 500 bp and 590 bp, 22 strains (21.6%) showed bands of 480 bp, 680 bp and 800 bp, 10 strains (9.8%) showed band of 500 bp and 580 bp, 8 strains (7.8%) showed bands of 500 bp, 720 bp and 800 bp, 7 strains (6.9%) showed bands of 500 bp, 580 bp, 700 bp and 800 bp, other 7 strains (6.9%) showed bands of 580 bp, 700 bp and 800 bp, where as other 5 strains (4.9%) showed bands of 480 bp, 500 bp and 580 bp, other 4 strains (3.9%) showed bands of 550 bp, 780 bp and 900 bp, 3 strains each (2.9)% showed 500 bp, 580 bp and 720 bp: and 550 bp, 800 bp and 900 bp respectively, 2 strains (1.9%) showed bands of 470 bp, 700 bp, 790 bp and 920 bp, whereas 1 strains (1.0%) showed bands of 550 bp 600 bp and 820 bp. Maximum numbers of strains showed a band pattern of 500 bp and 580 bp and were obtained from pus isolates.

In 100 animal-origin isolates 7 electrophoretic patterns were observed. Forty seven percent strains showed a band pattern of 900 bp, 17% strains showed bands of 650 bp and 700 bp, 13% strains showed bands of 590 bp, 680 bp and 700 bp, 8% strains showed bands of 600 bp, 7% strains showed bands of 650 bp, other 7% strains showed bands of 650 bp and 750 bp, whereas 1% strain showed bands of 500 bp and 820 bp.

The molecular typing of the clinical isolates and animal-origin isolates was carried out by Coa-RFLP, after the digestion with

Alu 1 enzyme as described by Goh *et al.*, 1992. A total 12 types of ***Coa* -RFLP** patterns were observed in **clinical isolates**. Majority of C***oa* -RFLP** patterns were observed in **clinical isolates**. Majority 30 (29.4%) of strains showed bands of 243 bp and 405 bp were classified in group XII followed by 22 strains (21.6%) in group III showing bands of 81 bp, 243 bp while 10 strains (9.8%) were characterized by distinctive bands pattern of 162 bp and 243 bp and were characterized in group VI. Eight strains (7.8%) having bands of 405 bp and 567 bp were categorized in group IX. Seven strains (6.9%) each showed bands of 81bp, 162 bp, 243 bp, 324 bp, 405 bp and 162 bp, 324 bp, 405 bp, respectively, and were classified in group I and group VIII. Five strains (4.9%) and 4 strains (3.9%) each showed bands of 81 bp, 162 bp: and 162 bp and 324 bp, respectively, were classified in group VII and group II, 3 strains (2.9%) each were classified in group IV and group X consisting of band patterns of 162 bp, 243 bp, 324 bp, 567 bp and 162 bp, 332 bp, 405 bp, respectively. Two strains (1.9%) showed band patterns of 162 bp, 243 bp, 405 bp belonged to group XI. However, 1 strain (1.0%) of group V showed band pattern of 243 bp, 324 bp. Heterogeneity was observed in specimens of a similar type. For example, *S. aureus* isolates from cervical swabs belonged to six different Coa-RFLP.

Among these 12 patterns, 7 group patterns, namely, group I, group II, group IV, group V, group VI, group VIII and group XI were noticed in MRSA isolates. Rest of the group patterns were in MSSA isolates. Briefly, out of 34 *Staphylococcus aureus* isolates from clinical specimens 7 (20.6%) belonged to *Coa*-RFLP pattern of groupI where majority of strains were resistant to six or more antibiotics; 10 strains (29.4%) belonged to *Coa*-RFLP group VI and 4 strains

(11.8%) of *Coa*-RFLP group II were also found resistant to six or more antibiotics. While 7 (20.6%) strains belonged to *Coa*- RFLP group VIII having resistance to five or more antibiotics. *Coa*-RFLP group IV and group XI having 3 strains (8.8%) and 2 (5.9%) strains respectively also showed resistance to five or more antibiotics, however, only one strain (2.9%) which belonged to *Coa*-RFLP group V showed resistance to five antibiotics.

In 100 **animal-origin isolates** 7 types of *coa* -RFLP were observed, maximum strains 47% were classified in group VI with bands of 405 bp followed by 17% strains characterized by distinctive band patterns of 243 bp and 324 bp in group V, 13% strains in group I with a band pattern of 162 bp, 250 bp and 405 bp, 8% strains showed bands of 81 bp and 324 bp and belonged to group VII. and 7% strains showed bands of 324 bp and were characterized as group II, 3% strains strain showed a band pattern of 162 bp and 405 bp classified in group IV. 1% strain of group III showed band patterns of 162 bp and 324 bp. Five each out of 29 raw milk isolates were classified in group VII and group II with band patterns of 81 bp, 324 bp and 324 bp. In the present study, *S. aureus* isolates from animal-origin samples and human clinical specimens were found to differ in their gene patterns whereas animal clinical specimens and animal-origin food samples were showed mixed Coa-RFLP gene patterns. This finding might provide a better understanding of the distribution of the *S. aureus* clones among human and animal-origin isolates and can be helpful in the control of *S. aureus* infections.

To appreciate the reproducibility of coa-RFLP the test were performed in duplicate. The reproducibility was good as similar results were obtained in both the tests.

Among these 7 patterns, 4 group patterns, namely, group II, group III, group I, and group VII, were noticed in 29 MRSA isolates from food samples. Rest of the group patterns were in MSSA isolates. Briefly, out of 29 *Staphylococcus aureus* isolates from food samples 8 (27.6%) belonged to *Coa* - RFLP pattern of group VII where majority of strains were resistant to six antibiotics. While 13 (44.8%) strains belonged to *Coa* - RFLP group I having resistance to five antibiotics. *Coa*-RFLP group III having resistance to seven or more antibiotics found in 7 strains (24.1%), however, only one strain (3.4%) showed resistance to three antibiotics were belonged to *Coa*-RFLP group III. Among these 7 patterns, 3 group patterns, namely, group IV, group V, and group VI were noticed in MSSA isolates. Antibiotic resistance patterns in relation to *Coa*-RFLP pattern could not be inferred in isolates obtained from food samples due to variability of resistance patterns; 40 different types of patterns. Majority 13 (44.8%) of the MRSA isolates showed *Coa*- RFLP pattern of group belonging to group I followed by group VII (27.6%) and group II (2.4%). However, no predominant resistance patterns were noticed.

We feel that *Coa*-RFLP technique could be incorporated as diagnostic tool to confirm the MRSA. It's also observed that the *Coa*-RFLP patterns of the MRSA strains were unique and distinct from the MSSA strains from both types of the isolates of *Staphylococcus aureus*. *Coa*-RFLP is performed with primers, homologous to a conserved region with in the *coa* gene, in order to

amplify the sequence encoding the C-terminals region of this molecule. Since the number of repetitive sequence varies within the *coa* gene, the resulting PCR products of individual strains can be of different lengths. Therefore we found different length and different patterns of *S. aureus* strains in *coa* RFLP. It is noted that there is extensive polymorphism with *coa* gene circulating in human and animal strains. We believe that the heterogeneity observed for the *coa* gene has a potential discriminatory power for further epidemiological studies of medical and veterinary importance.

For the detection of enterotoxin genes, namely enterotoxin A, enterotoxin B and enterotoxin C, PCR assay was used to amplify specific base pair products. A total of 202 isolates (102 human clinical isolates and 100 animal-origin isolates) were tested for the production of enterotoxin A, enterotoxin B and enterotoxin C. For the enterotoxin A and enterotoxin B modified method of Johnson *et al.* (1991) was used, which gave 120 bp and 478 bp gene specific products respectively while the modified method of Chen *et al.* (2001) used to amplify enterotoxin gene C which showed a gene specific 234 bp product.

Briefly, of the 102 **human clinical isolates** three were found enterotoxigenic, out of which one stool sample showed presence of enterotoxin A, 1 from vomitus sample showed enterotoxin B while one urine sample showed presence of enterotoxin C.

Out of 100 **animal-origin isolates** a total of 13 were found enterotoxogenic. Out of which 2 were positive for enterotoxin A (1 from raw milk sample and 1 from buffalo meat); 3 were positive for enterotoxin B (1 from raw milk sample, 1 from a sweet and 1 from cottage cheese) and 8 were found positive for enterotoxin C (1 from raw milk, 1 from paneer, 3 from goat meat and 3 from buffalo meat).

This is amongst the premier report regarding the prevalent enterotoxins in Indian *Staphylococcus aureus* and especially in clinical isolates. The phenotypic and genotypic results of the present study might help to understand the distribution of prevalent *S. aureus* clones in clinical and food isolates which can be the base to investigate and control the *Staphylococcus aureus* infections.

Conclusions:

On the basis of this study, following **conclusions** were drawn:

- 98.03% isolates from human clinical specimens and 98% isolates from animal-origin samples were positive for tube coagulase test, slide coagulase test and coagulase-PCR. Remaining 4 (1.98%) isolates were positive by tube coagulase test and coagulase-PCR, but missed detection by slide coagulase test.
- Multidrug resistance was noticed in both clinical and animal-origin isolates.
- None of the isolate was found resistant to vancomycin and teicoplanin in both human clinical and animal-origin isolates.
- Methicillin-resistance was found in 33.33% of clinical isolates and 29% of animal-origin isolates.
- Phage typing of the clinical isolates showed that only 70.58% clinical isolates were typeable using conventional phages at 1 RTD and at 100 RTD including 10 methicillin-producers. Amongst these, maximum number (35.3%) of isolates belonged to phage group III.
- Majority of the MRSA isolates were found non-typeable by conventional sets of phages. By using a set of MRSA phages they showed a typeability of 41.8% (14/34).
- In animal-origin isolates, only 2% isolates were found typeable using MRSA phages at 1 RTD and 100 RTD both of them belonged to group II.
- Disc diffusion, screen agar plate and polymerase chain reaction were used for detection of methicillin-resistance. MRSA were

detected in clinical isolates by disc diffusion method in 32.35%, by screen plate agar method in 31.41% and by PCR in 33.33%.

- One *mec* A positive isolate missed detection by disc diffusion while 2 isolates missed detection by screen agar plate method.
- 29% isolates from animal-origin samples were identified as MRSA by PCR, 27% by disc diffusion method and 26% by oxacillin agar plate method.
- PCR of MRSA seem to be promising for early and reliable identification of MRSA.
- All the human clinical and animal-origin isolates tested were positive for nuc gene.
- 12 Coa-RFLP patterns were observed in human clinical isolates.
- In 100 animal-origin isolates 7 types of *coa* –RFLP patterns were observed.
- In animal-origin samples and human clinical specimens different gene patterns were found.
- In animal clinical specimens and animal-origin food samples mixed Coa-RFLP gene patterns were observed.
- Coa-RFLP patterns were different for human clinical and animal-origin isolates.
- Coa-RFLP results suggest a divergence between *Staphylococcus aureus* isolates of human and bovine origin.
- Results of coagulase gene typing demonstrated that the MRSA and MSSA strains from clinical specimens could be grouped into 7 and

5 Coa-RFLP patterns, respectively. However, in animal-origin isolates 4 from MRSA and 3 from MSSA Coa- RFLP patterns were observed.

- The MRSA and MSSA strains did not share similar PCR-RFLP patterns. MRSA gave distinct *Coa*-RFLP patterns as opposed to those of MSSA.
- The ability of the PCR-RFLP typing method of the coagulase gene to differentiate between MRSA and MSSA in human clinical and animal-origin isolates was also noticed. It offers an attractive option to be considered for rapid epidemiological analysis of *S. aureus* strains.
- 102 human clinical isolates were tested for commonly countered enterotoxins; three of them were found enterotoxin producers, out of which 1 was positive for enterotoxin A, 1 for enterotoxin B and the other 1 for enterotoxin C.
- Out of 100 animal-origin isolates, a total of 13 were found enterotoxogenic. Of which 2% were positive for enterotoxin A; 3% were positive for enterotoxin B and 8% were found positive for enterotoxin C.
- Among the 16 enterotoxogenic *Staphylococcus aureus* isolates, 13 different antibiotic resistance patterns were observed in this study. All of the enterotoxogenic isolates from human clinical specimens were found resistant to methicillin. However all of the strains exhibited different antibiotic resistance patterns.
- In the remaining 13% enterotoxogenic animal-origin isolates 10 different antibiotic resistance patterns were observed. A total of 8% of them showed methicillin-resistance. Out of which, methicillin

resistance was noticed in 2% isolates, each, producing enterotoxin A and enterotoxin B, while in 4% isolates producing enterotoxin C.

In a nutshell diversity between human clinical and animal-origin isolates of *Staphylococcus aureus* was noticed and that the incidence of methicillin resistance was quite high in this collection of isolates. Concomitant high resistance to other classes of antibiotics was also noted. Phage typing was found to be of low discriminatory value whereas Coa-RFLP could discriminates a fairly large numbers of bacterial isolates and suggest that Coa-RFLP could be used as an epidemiological typing method for *Staphylococcus aureus*. The enterotoxins A, B and C were detected in our collection of human clinical, animal-origin food and animal clinical (raw milk) isolates. However, in animal-origin food isolates enterotoxin C was the predominant type.

A continuous surveillance on resistance patterns and characterization of *Staphylococcus aureus* in understanding new and emerging trends in India is of utmost importance for the formulation of infection control policies.

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APPENDIX

A) Reagents for genomic DNA extraction

1. Sodium dodecyl sulphate (10%)

SDS	10.0 gm
Deionized water	100.0 ml

Warmed to dissolve completely and adjusted pH to 7.0

2. Proteinase K 20 mg/ml

Proteinase K	20.0 mg
Distilled Water	1 0 ml

Stored at -20°C in small aliquots

3. Tris HCl (1m: pH 8.0)

Tris base	121.1 gm

Dissolved in 800 ml water, adjusted the pH to 8.0 by adding concentrated HC1. Made the volume to 1000 ml. Autoclaved and stored at 4°C

4. Tris EDTA (TE buffer pH 8.0)

TrisHCl	10 mM
EDTA	1 mM

5. 0.5 M EDTA (pH 8.0)

EDTA	18.612 gm
Distilled water	100.0 ml

B) Reagents for agarose gel electrophoresis

6. Tris-borate (EDTA BE) buffer (5x)

Tris base	54.0 gm
Boric acid	27.5 gm
EDTA (0.5 M; pH 8.0)	20.0 ml
Distilled water	1000 ml

7. Ethidium bromide

Ethidium bromide	100.0 mg
Distilled Water	1.0 ml

8. Loading dye (6x)

Orange G	0.2%
Bromophenol blue	0 05%
Glycerol	60%
EDTA	60 mM
Stored at	4 °C

C) General purpose solutions

9. *Phosphate buffered saline (pH 7.2)

Sodium chloride	8.0 gm
Potassium chloride	0 02 gm
Disodium hydrogen Phosphate (anhydrous)	1 15 gm
Potassium dihydrogen phosphate	0 20 gm
Distilled water	1000 ml

10. Normal saline solution

Sodium chloride	8.5 gm/ l
Distilled water	1000 ml

- Sterilized by autoclaving at 15 psi, 121°C for 15 min and stored at 4°C until used.
- The above materials autoclave at 121°C for 15 min and allowed to cool to 50°C -60°C and poured into petridishes.

Media and Reagents

11. Baird Parker Agar (PH 7.0)

Peptone	10.0 gm
Beef Extract	5.0 gm
Yeast extract	1.0 gm
Glycine	12.0 gm
Sodium pyruvate	10.0 gm
Lithium Chloride	5.0 gm
Agar	20.0 gm
Distilled Water	950 ml

The above base autoclaved and allowed to cool to 40-50°C and then about 50ml concentrated egg yolk emulsion and 3ml sterile 3.5% potassium tellurite solution added properly mixed poured in petridishes.

12. Oxidation – Fermentation Medium (g m/ml)

Peptone	2.0 gm
Sodium Chloride	5.0 gm
Dipotassium Hydrogen Phosphate	0.3 gm
Agar	3.0 gm
Bromothymol Blue (1% aqueous solution)	3.0 ml
Distilled Water	1000ml

The pH was adjusted to 7.1 before adding of the bromothymol blue and then medium was autoclaved in a flask at 121°C for 30 minutes. Then 0.5 ml, 1% carbohydrate solution (pre-sterilized) was added to medium aseptically. The medium was then tubed to a depth of about 4 cm.

13. Brain heart infusion Broth (pH 7.4+0.2)

Peptic digest of animal tissue	10.0 gm
Calf Brain Infusion (solids)	12.5 gm
Beef Heart Infusion (solids)	5.0gm
Dextrose	2.0 gm
Sodium chloride	5.0 gm
Di-Sodium phosphate	2.5 gm
Distilled Water	1000 ml.

14. 5 % Sheep blood agar

Base: blood agar (Hi Media)	5.0 gm
Distilled water	100ml

Dissolved the ingredients by boiling and autoclave at 121°C for 15 min
For complete medium: the autoclaved base was a lowed to cool to 46- 48°C and then add 10ml of aseptically collected defribinated sheep blood was added properly mixed and pour into petriplates.

15. Nutrient broth

Lean meat, ox heart or beef	500.0 gm
Peptone	10- 20.0 gm
Sodium chloride	5.0 gm
Distilled water	1 liter

The above materials autoclave at 121°C for 15 min and allowed to cool to 50 – 60 °C and poured into petridishes.

16. Nutrient agar:

Nutrient broth	1000ml
Agar	20.0gm

The above materials autoclave at 121°C for 15 min and allowed to cool to 50 – 60 °C and poured into petridishes.

17. DNAse agar (pH 7.3)

Tryptose	2.0gm
Deoxyribonucleic acid	2.0gm
Sodium chloride	5.0gm
Agar powder	12.0gm
Toludine blue	0.15 %

The above materials autoclave at 121°C for 15 min and allowed to cool to 50 – 60 °C and poured into petridishes.

18. Mueller – Hinton agar (pH 7.4)

Beef infusion	300.0ml
Casein hydrolysate	17.5gm
Starch	1.5gm
Agar	10.0gm
Distilled water	1 liter

The above materials autoclave at 121°C for 15 min and allowed to cool to 50 – 60 °C and poured into petridishes.

19. Mueller – Hinton agar with 5% Nacl (pH 7.4)

Mueller-Hinton Agar (Hi Media)	3.8 gm
Sodium chloride	5.0 gm
Distilled water	100.0 ml

The above materials autoclave at 121°C for 15 min and allowed to cool to 50 – 60 °C and poured into petridishes.

20. Phenolphthalein phosphate agar (pH 7.4)

Peptic digest of animal tissue	5.0gm
Beef extract	3.0gm
Sodium chloride	5.0gm
Sodium phenolphthalein phosphate	0.012gm
Agar	15.0gm
Distilled water	1000ml

The above materials autoclave at 121°C for 15 min and allowed to cool to 50 – 60 °C and poured into petridishes.

21. Voges-Proskauer

Peptone	5.0 gm
Dipotassium hydrogen phosphate (K 2 HPO4)	5.0gm
Distilled water	1000ml
Glucose 10% solution (sterilized separately)	50 ml

Peptone and phosphate were dissolved; pH was adjusted to 7.6; filtered and then dispensed in 5.0 ml amounts in test tubes and sterilized at 121 C for 15 minutes. Glucose solution was sterilized by filtrations and 0.25 ml was added to each tube (final concentration 0.5%).

22. Triptone soya broth (pH 7.3 + 0.2)

Casein enzyme hydrolysate	17.0gm
Peptic digest of Soya bean meal	3.0gm
Sodium chloride	5.0gm
Di- potassium hydrogen phosphate	2.5gm
Dextrose	2.5gm
Distilled water	1000ml

The above materials autoclave at 121°C for 15 min and allowed to cool to 50 – 60 °C and poured into petridishes.